5 Beta-Cholestan-3 Beta-ol
An Indicator of Fecal Pollution

By

CLIFF JOSEPH KIRCHMER

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
1971

ACKNOWLEDGEMENTS

I wish to dedicate this work to my father, who although financially unable himself to finish high school, is largely responsible for the opportunities of higher education that I have received.

Affectionate thanks to my wife, María Luisa, who, by providing a happy home, has established the psychological background without which this work would never have been possible.

Deepest thanks to my committee chairman, Dr. J. Edward Singley, for his advise as well as for the confidence he has shown in me throughout this study.

A special thank you to my friend Mr. Ryosuke Miura who performed the microbiological tests.

Thanks to the members of my committee, Professor T. deS. Furman, Dr. Jackson Fox, Dr. Patrick Brezonik, and Dr. James Winefordner for their advise and encouragement in both the research and preparation of this manuscript.

Appreciation is extended to Drs. Jaime Chiang and Walter Gaete, who through their example, conveyed to me the real meaning of higher education.

Particular thanks to Mrs. Nancy Townsend who not only typed the dissertation, but did so critically and professionally, helping to eliminate many of the author's errors. Appreciation is extended to Mr. Damon Hooten, who prepared the drawings.

The financial support of the Environmental Protection Agency, Water Quality Office, under EPA Grant number 16020 EVG was instrumental in the prosecution of this study as was the interest shown by Mr. William Donaldson of the Water Quality Laboratory, Athens, Georgia.

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Cliff Joseph Kirchmer

August, 1971

Chairman: J. Edward Singley, Ph.D.
Major Department: Environmental Engineering

Total coliform and fecal coliform organisms have been used in the past as indicators of fecal pollution. However, these indicators have shown many inadequacies, so that the National Technical Advisory Subcommittee on Public Water has stated that "It is essential that raw-water sources be judged as to suitability by other measures and criteria than coliform organism concentrations."

The study reported in this dissertation was an investigation of a chemical indicator of fecal pollution, 5β-cholestan-3β-ol (coprostanol). Experiments were performed to improve and shorten existing methods of analysis, as well as to determine the applicability of coprostanol as a water quality indicator.

It was found that the free sterol could be chromatographed by using an acid-washed, silanized, diatomaceous earth as column support for SE-30. Improved cleanup of water samples was obtained by washing hexane extracts with acetonitrile saturated with hexane.

Results indicated that coprostanol present in sewage is present primarily as the free sterol, with only approximately 10 percent present in the form of esters.

Evidence was presented indicating that the removal of coprostanol by sewage treatment is primarily due to biodegradation by microorganisms. Determinations were made on the decay rate of coprostanol in laboratory samples of both chlorinated and unchlorinated sewage effluents. It was shown that samples must be preserved with sulfuric acid or by cooling to avoid biodegradation of coprostanol by microorganisms while in storage.

Field surveys were made on the receiving streams and lakes of two sewage treatment plants in Gainesville, Florida. Free sterols were measured and found satisfactory as indicators. No correlation between coliform indicators and coprostanol was found in these surveys, primarily due to the effect of chlorination on coliform organisms.

It was concluded that free coprostanol can be used as an indicator of fecal pollution in those cases where the fecal coliform tests are inadequate.

CHAPTER I

INTRODUCTION

At the beginning of this century a Mississippi jurist said, "It is not necessary to weigh with care the testimony of experts—any common mortal knows when water is *fit to drink*."[1] Perhaps even at that time the statement was an exaggeration, but certainly now most recognize that experts are necessary not only to determine when water is fit to drink but even to determine if it may be used for bathing. Our demand for water has grown rapidly in the past half century and promises to increase even more rapidly in the future. Gross withdrawal of water in the United States amounted to 302.3 billion gallons per day in 1954 and is expected to rise to 888.7 billion gallons per day by the year 2000.[2] Increased use means that the danger of pollution is also increased. In his message to Congress on 23 February 1961, President Kennedy said, "Pollution of our country's rivers and streams has—as a result of our rapid population and industrial growth and change—reached alarming proportions. To meet all needs—domestic, agricultural, industrial, recreational—we shall have to use and reuse the same water, maintaining quality as well as quantity. In many areas we need new sources of supply—but in all areas we must protect the supplies we have."[3]

Water quality must be considered together with its intended use. We may consider water quality as a continuous spectrum with potable water at one end and wastewater effluent at the other end. Between these two extremes fall quality criteria for bathing, fishing,

shellfish harvesting, irrigation, and industrial use. To characterize a water's suitability for a given use, we must have some means of measuring its quality.

Total coliform and fecal coliform have been used as measures of water quality for uses ranging from shellfish waters to public water supplies. Unfortunately, coliform organisms may originate from sources other than feces. Even the membrane filter procedure for fecal coliform gives only 93 percent accuracy for differentiating between coliforms of warm-blooded animals and coliforms from other sources.[4] To obtain good results, one must analyze for coliform within 30 hours after sampling. A minimum of 24 hours are needed for incubation, so there is a delay between sampling and knowledge of fecal pollution. Chlorinated sewage effluents often show low coliform counts even though water quality is poor. Because of the weaknesses of the coliform tests, research has continued in the search for indicators of fecal pollution.

An indicator which shows promise is 5β-cholestan-3β-ol (coprostanol).[5,6,7] This sterol is apparently found only in the feces of mammals, including man, and therefore is a specific fecal indicator.

Procedures used for isolating and measuring coprostanol in wastewater plant effluents and surface waters are somewhat time-consuming. It has been estimated that two men could analyze only 10 samples per day for coprostanol.[5] Little is known about whether the removal of coprostanol in water is a chemical, biological or physical process. Nor is it known what rate of removal is expected. While analytical sensitivity is good, improvement in sensitivity would enable more widespread use of the test. Only one field survey of coprostanol has been reported.[5]

This study was undertaken to answer some of the problems that have arisen as a result of previous research on coprostanol in water and wastewater. Emphasis has been placed on methods for increasing the speed of analysis. Experiments were also performed to determine the mode and rate of removal of coprostanol in sewage effluent. Finally, field surveys of coprostanol were performed to determine concentrations of coprostanol in the aquatic environment as well as to determine any possible relationships between microbiological indicators and coprostanol.

CHAPTER II

INDICATORS OF FECAL POLLUTION AND THEIR RATIONAL BASES

Introduction

It is important for public health reasons to have some means of determining whether a water has been polluted with feces from humans or other animals, since feces are a prime source of pathogenic organisms. The most common genera of pathogenic organisms found in water are: Salmonella, Shigella, Vibrio, Mycobacterium, Pasteurella, and Leptospira.[8] In addition, enteric viruses have been shown to be present in some waters.[9]

It is possible to detect many pathogenic organisms in water. However, the techniques necessary for their detection are, in general, complicated, time-consuming, and often present a health risk to the analyst. In addition, failure to show that a particular pathogenic organism is present does not ensure that other pathogenic organisms are also absent. Among the pathogenic organisms, only Salmonella[10] and fecal streptococci[11,12,13] have been used as indicators of pollution.

Because of the problems of isolating pathogenic organisms, certain non-pathogenic bacteria have been used as indicators of fecal pollution. No organism has been found which could be classified as a perfect indicator. One group, the coliform group, has been used, but has shown many inadequacies, enumerated in the next section, which has

led to continued research for other indicators, both biological and chemical.

Biological Indicators

Escherich in 1885 was the first to describe Bacillus coli (now referred to as the "coliform group") as an indicator of fecal pollution.[14] The coliform group, however, is quite complicated, and there followed a rather long period in which bacteriologists developed methods and interpreted results for coliform analyses. As a result of this work, we now have certain standard methods for detecting coliform organisms.

Escherichia coli is characteristically present in human and animal intestines and, therefore, is a potentially good indicator of fecal pollution.[14,15,16,17] Other members of the coliform group such as Aerobacter aerogenes and Aerobacter cloacal [known as the intermediate-aerogenes-cloacal (I.A.C.) subgroups] have less sanitary significance since they can originate from sources other than feces. They have been found,for instance, on various types of vegetation,[18,19] in materials used in joints and valves of pumps and in pipelines,[20,21] in soils,[22,23,24] and in the guts of cold-blooded animals.[25,26,27] The I.A.C. subgroups may be found in feces, but usually in smaller numbers than E. coli.

The coliform group of organisms is operationally defined according to the method of analysis. When the multiple-tube fermentation technique is used for analysis, the coliform group is said to comprise all of the aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria which ferment lactose with gas

formation within 48 hours at 35°C. When applied to the membrane filter technique, the coliform group is defined as comprising all the aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria which produce a dark colony with a metallic sheen within 24 hours on an Endo-type medium containing lactose.[28]

The coliform group as measured by the multiple-tube fermentation technique, therefore, may not be exactly the same as the coliform group as measured by the membrane-filter technique. Nonetheless, both techniques are considered valid methods for determining the sanitary quality of the water.

Elevated temperature tests as well as membrane filter procedures have been developed which differentiate between coliforms of fecal origin and coliforms from other sources. It should be emphasized that a perfect procedure for distinguishing between fecal and non-fecal coliforms has not as yet been developed. This failure may result from a number of factors including the difficulty of obtaining coliform bacteria from known sources for evaluation of the test procedure (it is difficult, for example, to be certain that a soil sample is uncontaminated), limitation of methods, and lack of biochemical uniformity among the coliform groups.

Common Chemical Indicators

It has long been recognized that certain common chemical tests give evidence of fecal pollution, even though they are not specific indicators. These tests include, for example, free ammonia, nitrites, chloride, carbonaceous matter as BOD or COD, and dissolved oxygen.

It has been suggested that water containing more than 0.1 mg/l of ammonia should be suspect of recent pollution.[29,30] The World Health Organization European Drinking Water Standards set a recommended limit of 0.5 mg/l as NH_4^+.[31] Nitrite is usually formed in water by the action of bacteria on ammonia and organic nitrogen, but is rarely present in large concentrations, since it is quickly oxidized to nitrate, the thermodynamically stable form of nitrogen in oxygen containing waters. Nitrate may also indicate the presence of pollution when it is present in concentrations higher than normal for the locality.

Chloride is normally present in a higher concentration in sewage than in receiving waters. Humans normally excrete an average of 6 g of chloride per person per day which results in an increase of chloride in sewage of about 15 mg/l above that of the carriage water.[32] The increase in chloride concentration in a stream then may be indicative of sewage pollution.

Sewage often has a high concentration of carbonaceous material. The amount of carbonaceous material can be measured by the operationally defined BOD or COD test, and used as an indicator of the extent of pollution of water. However, certain naturally occurring, unpolluted waters, may have high CODs and BODs due to the presence of natural organic material in the water.

Finally, dissolved oxygen may be used as an indicator of pollution by sewage. Where pollution has occurred, the oxygen is depleted as a result of aerobic biodegradation of the organic wastes.

Each of the above substances may be used to indicate pollution. However, a better indication of pollution could be obtained by examining

the water for more than one substance. For example, the presence of nitrite indicates possible sewage pollution, but if the nitrite is also present with ammonia and high chloride concentration, the conclusion of sewage pollution is more certain.

It has been said that "the state of change is the state of danger."[33] Analyses of the above substances must always be accompanied by an awareness on the part of the analyst of the normal values to be expected for the water sample. Any change in the analysis of a water sample over a period of time may indicate danger.

For example, a summary of quality changes that occur in water is well summarized by Bunch and Ettinger.[34] Tap water and sewage effluent for five cities in Ohio were analyzed for various constituents. Data from this study are given in Tables 1 and 2. Average values in Table 2 represent values for five cities.

TABLE 1
Cities Studied, Water Quality Depreciation by Municipal Use

City	Population	Waste Flow MGD	Treatment Process	Water Source	Water Softening
Batavia	1,440	0.1	Trickling Filter	River	Yes
Dayton	262,000	39.86	Trickling Filter	Wells	Yes
Hamilton	57,950	8.0	Activated Sludge	Wells	Yes
Lebanon	4,620	0.65	Activated Sludge	Wells	No
Loveland	2,150	0.20	Trickling Filter	Wells	No

All of the indicators discussed in this section suffer from the fact that none are specific indicators of fecal pollution. For example, ammonia may come from industrial waste water, chloride may be present in the waste water from a food-processing factory, carbonaceous material may come from decaying vegetation, etc. Rather than specifically

TABLE 2

Water Quality Depreciation by Municipal Use

	Tap Water Average (mg/l)*	Sewage Effluent Average (mg/l)*	Increment (mg/l)*
COD-unfiltered	6.0	149	143
COD-filtered		101	95
COD-filtered, corrected for Cl^-	2.0	84	82
Anionic detergents	20.02	7.4	7.4
Hydroxylated aromatic (tannic acid)	0.05	1.7	1.6
Carbohydrates (glucose)	0.05	2.5	2.4
Reducing sugars (glucose)	<0.005	<0.005	
Organic nitrogen (N)	0.07	2.3	2.2
Nitrate (N)	0.67	4.1	3.5
Nitrite (N)	0.031	0.33	0.3
Ammonia (N)	<0.02	16.1	16.1
Total Nitrogen (N)			22.0
Total alkalinity ($CaCO_3$)	141	263	122
Calcium (Ca^{++})	52	75	23
Magnesium (Mg^{++})	15	22	7
Potassium (K^+)	1.9	11.2	9.3
Sodium (Na^+)	13.5	70	57
Phosphate ($PO_4^{\equiv}$)			
Total	0.043	24.3	24.3
Ortho	0.011	22.8	22.8
Sulfate	68	101	33
Chloride	15.1	70.9	56

TABLE 2—*Continued*

	Tap Water Average (mg/l)*	Sewage Effluent Average (mg/l)*	Increment (mg/l)*
Residue 105°C	256	547	291
Residue 600°C	198	420	222
Loss on ignition	58	127	69
pH*	8.3	7.5	-0.8
Specific conductance $\left(\frac{\text{micromhos}}{\text{cm}}\right)$*	422	916	494

*Units for pH and Specific conductance are not in mg/l.

indicating pollution, these indicators must be used in conjunction with intuition and knowledge of the other environmental factors that are involved.

Uric Acid

Uric acid has been used as a pollution indicator.[35,36] The amount of uric acid in a specimen is measured by observing the reduction in absorbance at 292 mμ wavelength resulting from its oxidation by the enzyme uricase. Uric acid is the principal vehicle of nitrogen excretion for reptiles, birds, and insects, and is the end-product of purine metabolism in man, the higher apes, and Dalmation dogs.[37] However, 95 percent or more of the uric acid is degraded by conventional treatment processes and no uric acid could be detected in the Ohio River even in samples taken near the outfall of the Little Miami River Sewage Plant.[35] This indicates that only recent pollution of a stream with raw waste water could be detected by use of uric acid.

Cholesterol

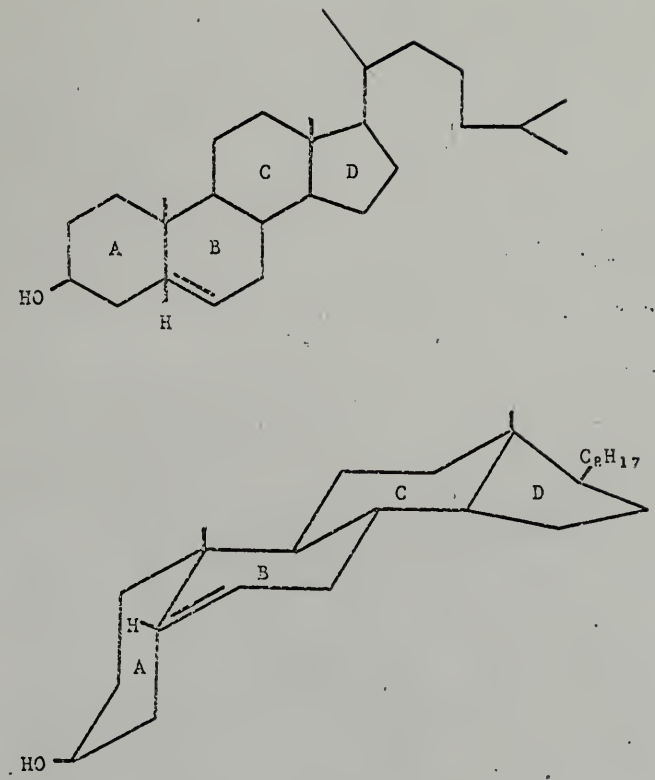
Cholesterol has been suggested as an indicator of fecal pollution, and the concentration of cholesterol in natural waters has been measured.[5] Since it is known that cholesterol in surface waters could come not only from excreta, but also from eggs, milk, lard, wool grease, etc. cholesterol can not be considered a specific indicator of fecal pollution. In seawater, cholesterol is evidently present naturally at concentrations close to its water solubility.[85,86] The origin of this cholesterol is unknown. Nonetheless, the use of cholesterol as an indicator in fresh water may be useful; although it

would give false positive results on some tests, it would not give false negative results, a more serious problem in assessing water safety.

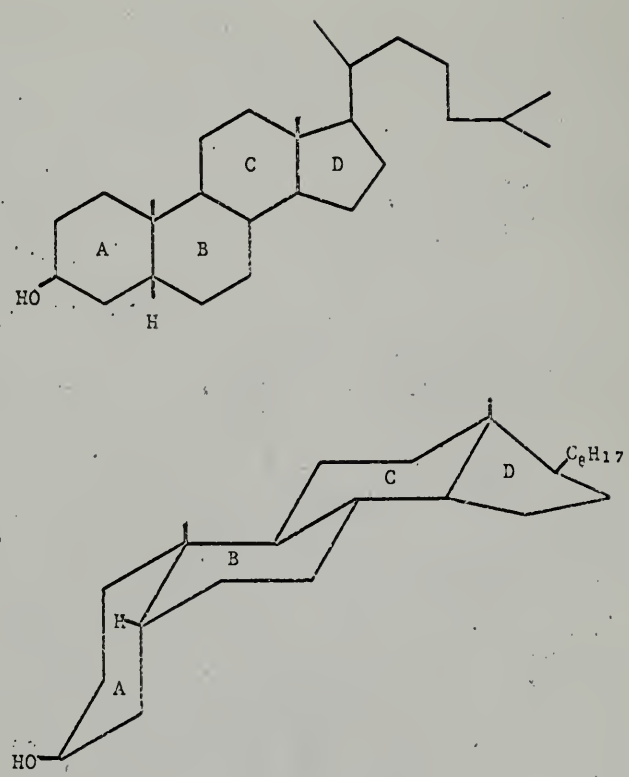
Coprostanol

Coprostanol, or 5β-cholestan-3β-ol, has shown promise as an indicator of fecal pollution.[5,6,7] This sterol is a characteristic fecal organic compound which is found in the feces of higher animals, including man.[38] Recently it has been claimed that coprostanol is present in whale oil.[39] Nonetheless, for all practical purposes, the only apparent source of coprostanol is the feces of the higher animals. Under normal conditions, coprostanol is the major fecal neutral steroid. In addition, the fecal neutral steroid fraction contains varying but usually small amounts of the following steroids of endogenous origin: cholesta-5,7-dien-3β-ol (7-dehydrocholesterol), cholesterol, 5α-cholest-7-en-3β-ol (lathosterol), 5β-cholest-7-en-3β-ol,4α-methyl-5α-cholest-7-en-3β-ol (methostenol), 5α-cholestan-3β-ol (cholestanol), 5β-cholestan-3α-ol, 5α-cholestan-3-one, and 5β-cholestan-3-one.[40] It is claimed that a small part of the sterols is present in the form of esters.[41,42,43]

The microbial conversion of cholesterol to coprostanol in the large intestines of mammals has been demonstrated[44,45] and the mechanism of the conversion process has been investigated.[46,47,48] The major reaction mechanism has been found to be a direct saturation of the double bond mediated by intestinal microorganisms. The pathway for formation of coprostanol esters has been investigated and the conclusion was reached that the main pathway was an esterification of free 5β-cholestan-3β-ol and not a hydrogenation of cholesteryl esters.[49] Figure 1 shows the structures of cholesterol and coprostanol.



Cholesterol
(Cholest-5-en-3β-ol)



Coprostanol
(5β-cholestan-3β-ol)

Fig. 1 - Planar and Perspective Representations of Cholesterol and Coprostanol

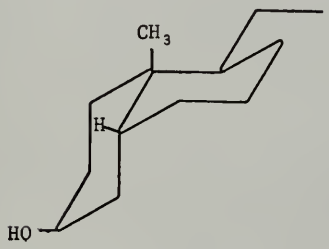
Studies have shown that the fecal excretion of neutral fecal steroids in human subjects fed standardized diets containing either saturated or unsaturated fat ranges from 500-700 mg per day,[50,51,52,53,54,55] the majority of which consists of coprostanol, cholesterol, and their esters. However, studies of the concentration of coprostanol in sewage indicate concentrations of coprostanol corresponding to about 2 grams of coprostanol per capita per day.[5]

Coprostanol was first isolated from human feces by Austin Flint, Jr. in 1862. The pharmacologist von Bondzyński characterized the sterol as an alcohol of the formula $C_{27}H_{48}O$ and gave it the name coprosterol (from the Greek Kopros, dung). The name was later changed to coprostanol for uniformity of nomenclature.

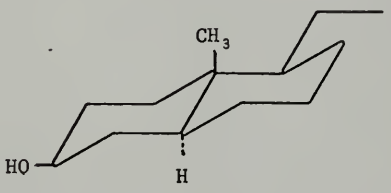
Coprostanol is the C_5-epimer of cholestanol. The typical saturated sterols found in nature are A/B trans or 5 α compounds, and the only exception to this rule is coprostanol. It is said that epicoprostanol (5β-cholestan-3α-ol) is thermodynamically more stable than coprostanol because of steric strain from α axial hydrogens.[56] Structures of cholestanol, coprostanol, and epicoprostanol are shown in Figure 2.

Coprostanol is a white crystalline solid at room temperature, showing a melting point of 101°C. A variety of acids form esters with coprostanol, including the acetate, propionate, and benzoate which exhibit melting points of 89-90°, 99-100°, and 124-5°C respectively. It is very soluble in ethanol, ether, benzene, and chloroform, and slightly soluble in methanol.[57] It is only very slightly soluble in water. No values for its solubility in water are given in the literature, but values for a structurally similar sterol, cholesterol, have been

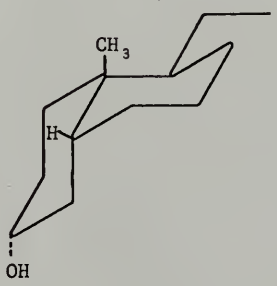
given as 2.6×10^{-8} g/ml at 30.0°C, which corresponds to 26 µg/liter.[58] Coprostanol would be expected to show a similar solubility in distilled water. However, in primary sewage effluent, values have been reported as high as 260 µg/liter for cholesterol and 750 µg/liter for coprostanol,[5] which indicate that the amounts of cholesterol and coprostanol found in sewage are not limited by their solubilities and that much of the cholesterol and coprostanol is present combined with particulate matter.



Coprostanol
(5β-cholestan-3β-ol)



Cholestanol
(5α-cholestan-3β-ol)



Epicoprostanol
(5β-cholestan-3α-ol)

Fig. 2 - Structural Differences Between Coprostanol, Cholestanol, and Epicoprostanol

CHAPTER III

BACKGROUND MATERIAL ON THE ANALYSIS OF COPROSTANOL

Methods of Analysis

Coprostanol has been analyzed by colorimetric methods as well as by gas-liquid chromatography (GLC),[59,60,61,62] but the colorimetric methods suffer from a lack of sensitivity as well as the ever-present possibility of spectral interferences. Gerson[61] considers his colorimetric method, which employs a color reagent consisting of ferric choloride - acetic acid - concentrated sulphuric acid, not suitable for the determination of less than 75 µg of coprostanol. This amount would suffice for the analysis of coprostanol in 1-2 liters of primary treated sewage, but, in many cases, secondary treated sewage contains coprostanol in concentrations too low to be detected by Gerson's method. The absorption peak, measured at 440 mµ, may exhibit interferences, since sewage is a complex mixture containing many compounds that may absorb at 440 mµ. Thus, a cleanup step involving digitonide precipitation and/or thin-layer chromatography (TLC) would be necessary.

Despite the difficulties involved in developing a colorimetric method for detecting coprostanol in the environment, it would be well worth the effort. A colorimetric method for cholesterol that is capable of detecting amounts as small as 2 µg has been developed.[59] A similar method for coprostanol may be feasible. Advantages of a colorimetric

method would be more rapid analysis, less expensive equipment, and less technical training. It may be possible to develop a colorimetric test for total sterols, which could be used to measure the efficiency of sewage treatment processes, since coprostanol is removed in a properly designed and operated activated sludge plant.

Another possibility is the use of liquid-liquid chromatography (LLC) for the separation and analysis of coprostanol. LLC would seem to be a good separation method, but the detectors used in LLC are not as sensitive as those available for use with GLC.

This project has centered on GLC as the principal analytical tool for the analysis of coprostanol because of the excellent separation that is possible, as well as for the sensitivity of the method, which can detect concentrations of coprostanol less than 1 µg/liter in the environment.

Sampling Procedures

Samples have been taken in both glass[5,6] and plastic[7] containers. Glass containers would seem to be preferable since adsorption onto glass is less likely than adsorption onto plastic. None of the researchers have reported the preservation of water samples. Indeed, no mention has been made that preservation may be necessary. Bunch states that, whenever possible, extraction with hexane was performed on the day the sample was taken,[5] indicating that the concentrations of coprostanol may be changing with time.

Extraction and Cleanup

The three previous studies on the analysis of coprostanol in the environment have used slightly different techniques for extraction and cleanup.[5,6,7]

Murtaugh and Bunch were the first to analyze for coprostanol in the environment and used a procedure which consisted of extraction with hexane and cleanup by thin-layer chromatography (TLC).[5] A small amount of hydrochloric acid and sodium chloride was added to the samples in order to enhance recovery. Seventy percent ethanol was used to wash the hexane extracts and break up any hexane-water emulsions that may have formed. TLC plates were prepared by coating 8 × 8 inch glass plates with a 0.25 mm layer of methanol-washed silica gel G and activated one hour at 110°C. The chromatoplate was developed with a mixture of chloroform and ether in the ratio of 9:1. Sterols were located by covering that portion of the plate containing the unknown samples and spraying the reference standards with a 10 percent solution of phosphomolybdic acid in 95 percent ethanol. After heating at 100°C for 5 minutes, the sterols appeared as dark spots on a yellow background. R_f values for coprostanol and cholesterol were 0.65 and 0.50 respectively.

Smith and Gouron also extracted water samples with hexane and used a cleanup by TLC. TLC was performed using 20 × 20 cm and 20 × 40 cm chromatoplates coated with 0.25 mm thick silica gel HF_{254} (E. Merck, Darmstadt). Irrigation was done principally with benzene:ethyl acetate (3:2). Visualization was accomplished using ultraviolet light followed by spraying with 50 percent aqueous sulfuric acid.

Black, Singley, and Nordstrand[7] found it only necessary to extract water samples with chloroform, evaporate the solvent, and redissolve the residue in chloroform prior to analysis by gas-liquid chromatography (GLC). No cleanup by TLC was found necessary for the samples they analyzed.

Saponification

Some work has been done to determine the form of sterols present in feces.[64,65,66,67,68] Rosenfeld's work was the most quantitative. He analyzed seven samples taken from five subjects and found 30.07 percent of total coprostanol in the form of esters.[67] The experimental technique involved a preliminary separation by column chromatography on alumina, followed by a saponification of the esters and quantification by GLC.

On the basis of the evidence for the existence of coprostanol esters, Bunch and Gouron included a saponification step in the analytical procedure for coprostanol. Samples were refluxed for 3 hours in a solution of 7.5 percent potassium hydroxide in 70 percent ethanol after initial extraction with hexane and prior to cleanup by TLC.

Other investigators have omitted the saponification step.[6,7] No reason was given for the omission. Evidently, either the step was considered too time-consuming or the percent of esters was considered too small to need recovery.

Gas-Liquid Chromatography

The basic technique for gas-liquid chromatographic separation of the steroids was established by W. J. A. Vandenheuvel *et al.* in 1960. The technique used a column of 2-3 percent SE 30 on 80-100 mesh Chromosorb W. A number of steroid compounds including hydrocarbons, ketones, alcohols, ethers, and acetyl esters were found to separate at 222°C with no sign of decomposition.

The technique of Vandenheuvel was soon applied to the analysis of fecal neutral steroids.[70] Some investigators found that the use of trimethylsilyl ether derivatives of the sterols gave sharper peaks and increased resolution.[70,71] The method of Vandenheuvel has been used, with certain modifications, by all of the investigators of coprostanol in environmental water samples.

Murtaugh and Bunch used a 1/8 inch by 5 foot stainless steel column packed with 60-80 mesh Chromosorb W coated with 5 percent SE 30.[5] Oven temperature was 235°C and injector port and detector were both kept at 260°C. Helium carrier gas was used at a flow rate of 40 ml/minute. Trimethylsilyl ether derivatives of all samples were prepared prior to injection onto the column. Quantitative determinations were based on peak height compared to a standard curve prepared from known reference standards.

Smith and Gouron used a 6 mm by 1.83 m glass tube packed with 3 percent SE 30 on 80-100 mesh Gas-Chrom Q.[6] Analyses were conducted using Hewlett-Packard Corp. F + M Models 400 and 402 gas chromatographs equipped with hydrogen flame detector aystems. Column temperature was 230°C and injection port was maintained at 260°C. Nitrogen gas

was used as carrier at a flow rate of 20 ml/minute. Free sterols were chromatographed, but no quantitative determinations were made.

Black *et al.* used a 4 mm by 4 foot glass U tube packed with 3 percent OV17 on 60-80 mesh Gas Chrom Q. A Research Specialties Company Model 600 gas chromatograph equipped with a ^{90}Sr ionization detector was used for all analyses. Argon was used as carrier gas with inlet pressure being maintained at 30 lb/in^2. Column temperature was maintained at 240°C or programmed from 150-240°C, and the injection port was preheated to 270°C. Peak heights obtained from the chromatography of standard solutions were used for quantitative analysis. Free sterols were chromatographed and found satisfactory. However, for verification of coprostanol, silyl ether derivatives were prepared on column by bis(trimethylsilyl)acetamide.

Stability of Coprostanol

It is known that coprostanol decreases in concentration when sewage is subjected to biological treatment.[5] Coprostanol is also known to decrease with increasing distance from source of pollution in streams and rivers.[5,72] However, it is not known if the disappearance of coprostanol is due to physical, chemical, or biological factors. Smith has speculated that the removal of coprostanol in the activated sludge process is physical.[6]

It is quite likely that coprostanol is biodegraded in sewage treatment plants and streams since microorganisms can utilize such complex molecules as steroids for growth, as first demonstrated by Sohngen in 1913.[73] Indeed, Turfitt has shown that coprostanol is utilized by a number of strains of Proactinomyces isolated from 5 different soil types.[74] Coprostanol acetate and coprostanone were

also found to be utilized. A medium consisting of 50 ml of mineral salt solution and approximately 1 mg of steroid was innoculated with the soil organisms. Increased bacterial count or development of mold myceluim, together with alteration of the pH of the medium, was regarded as evidence of steroid utilization. Turfitt concluded that steroids generally, with a very few exceptions such as halogen-substituted derivatives, are attacked by Proactinomyces of soils, and these are clearly the predominant organisms in steroid decomposition.

Although it appears that no other studies on the utilization of coprostanol by microorganisms have been published, there are a number of publications on the utilization of cholesterol. Sohngen as well as Haag reported that species of Mycobacteria were capable of growing in a medium containing cholesterol as the only source of organic carbon.[73,75,76] Tak grew three species of soil Mycobacteria on cholesterol and demonstrated the actual disappearance of this compound from the medium.[77] Subsequently, a variety of bacteria and fungi have been shown to utilize sterols.[74,78,79,80,81,82,83]

A recent paper[84] described experiments performed to determine the cholesterol-decomposing ability of 1,589 microbial strains consisting of 276 bacteria, 132 actinomycetes, 905 molds, and 276 yeasts. It was found that 286 strains were able to oxidize more than 20 percent of the available cholesterol (0.1%) in seven days and 18 of those strains were capable of over 50 percent decomposition. No apparent correlation between the cholesterol-decomposing activity and the taxonomical characteristics of the microorganisms was found but it was found possible to divide the microorganisms into three groups according to their

degradation pattern of cholesterol. One group was found to give cholest-4-en-3-one as a degradation product, a second group gave cholesta-1,4-diene-3-one together with cholestenone in a fermentation mixture, and the third group produced no steroid intermediates in fermentation mixture with an appreciable disappearance of cholesterol.

Field Surveys

Only Murtaugh and Bunch have reported a survey of coprostanol in the environment.[5] They surveyed a stretch of the Little Miami River near the city of Loveland, Ohio, and found concentrations of coprostanol ranging from less than 0.02 µg/liter above the outfall of the Batavia, Ohio Treatment Plant to 5.0 µg/liter below the outfall of the Loveland, Ohio Treatment Plant. Another survey that is soon to be published reports concentrations of coprostanol in the Missouri, Mississippi, and Ohio River Basins.[72] Twenty-eight sampling points on a 443-mile stretch of the Missouri River gave mean values on 4 analyses ranging from 6 µg/liter to 797 µg/liter.

Others have reported finding coprostanol in the environment, although no attempts were made at complete surveys. Smith and Gouron in an essentially qualitative study, reported detecting coprostanol in Galveston Bay at a distance of up to 100 meters from a sewer outfall.[6] Black *et al.* found coprostanol in a small creek and also, significantly, in city well water in Gainesville, Florida.[7]

CHAPTER IV

EXPERIMENTAL

Chemical Materials

All solvents used in this study were reagent grade or its equivalent. Pesticide quality hexane (Matheson Coleman and Bell) was used as the extracting solvent. Reagent grade 95% ethanol as well as acetonitrile (Mallinckrodt Chemical) were used in the cleanup procedure.

Coprostanol was supplied in pure form by K + K Laboratories, Inc., Plainview, New York as well as by Applied Sciences, Inc., State College, Pennsylvania. Cholesterol was obtained from K + K Laboratories, Inc. and cholestane was purchased from Applied Science, Inc. Coprostanol was judged pure on the basis of melting point determinations as well as gas chromatographic data.

Membrane filter media (Bacto-m Endo Broth MF, Bacto-m FC Broth Base, and Bacto-Rosolic Acid) were purchased from Difco Laboratories, Detroit, Michigan. Disposable plastic petri dishes (48 × 8.5 mm) were obtained from Millipore Corporation, Bedford, Massachusetts (see Figure 3).

Three percent SE-30 on Gas Chrom Q served as liquid and support phase for gas chromatography. Three percent QF1 on Gas Chrom Q was used for confirmatory analyses. Gas Chrom Q is an acid washed, silanized, diatomaceous earth.

Fig. 3 - Author Writing Results for Coliform Analyses

Apparatus

Glass sampling bottles were used for all samples. Extraction was done in two liter separatory funnels equipped with Teflon stopcocks to avoid contamination from stopcock grease (see Figure 4).

Either a Büchi Evaporator (Rinco Instrument Company) or a Buchler Flash Evaporator (Buchler Instruments) were used to reduce the volume of hexane extracts. A 500 ml Kuderna-Danish Evaporative Concentrator equipped with a 5 ml receiver was used to collect the reduced volume of hexane (Ace Glass Company). With this apparatus (see Figure 5) it was possible to remove the 5 ml receiver with the concentrated hexane extract and thus avoid loss of extract on transfer.

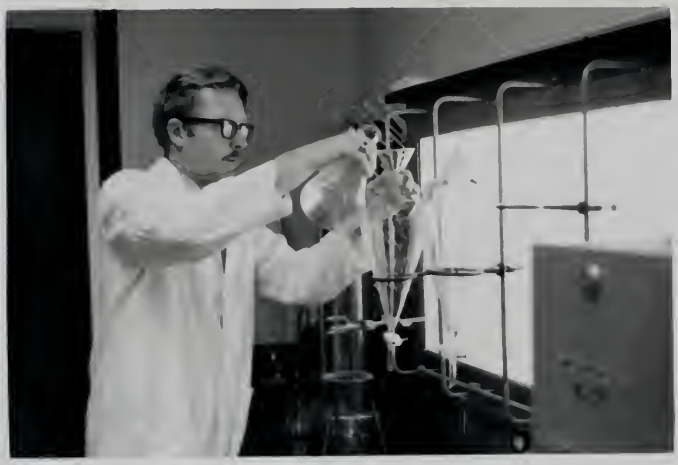


Fig. 4 - Author Transferring Water Sample to Two-Liter Separatory Funnel

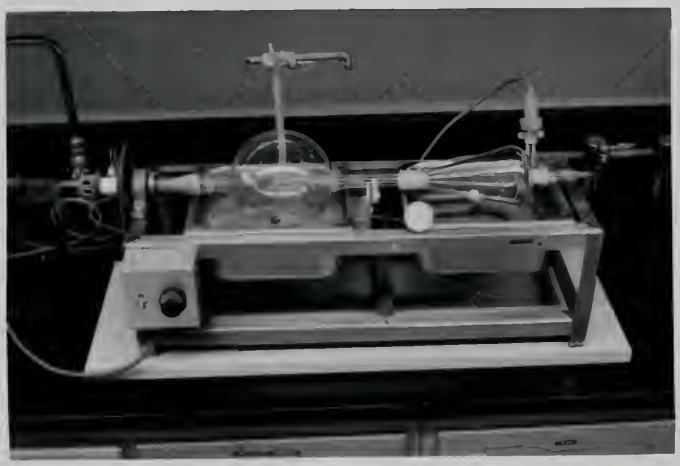


Fig. 5 - Flash Evaporator Apparatus with Kuderna-Danish Evaporative Concentrator Equipped with 5 ml Receiver

A 10 µl syringe (#701 syringe, Hamilton, Company, Whittier, California) was used for all gas chromatography. Six feet × 1/4 inch coiled glass columns (Applied Science) were employed in gas chromatography.

A F + M Corporation Model 810 gas chromatograph, equipped with both flame ionization and Ni^{63} electron capture detectors was used. The recorder was a Sargent Model SR, reading 1 millivolt full scale (see Figure 6).

Fig. 6 - Author, Together with Instruments Used in Gas Chromatography

Procedures

Most extractions of water samples were done in the following manner. Exceptions are noted in discussions of specific procedures. One liter water samples were generally taken, and 2 ml HCl (conc)

and 5 ml 20 percent NaCl were added. The solution was extracted with 2 50 ml portions of hexane. The hexane extracts were combined and washed with two 25 ml portions of 70 percent ethanol followed by two 25 ml portions of acetonitrile saturated with hexane. The hexane was reduced to near dryness on a flash evaporator. The Kuderna Danish Evaporative Concentrator was then washed with a small amount of hexane and the concentrated extract was collected in the 5 ml receiver. The receiver was removed and the hexane was evaporated to dryness with a stream of dry air. After redissolving in a known amount of hexane, the sample was ready for gas chromatography.

To correct injection errors, the following technique of filling the microsyringe was used. First a small amount of air was drawn into the syringe. The syringe was held down and then the sample liquid was drawn in followed by more air. This gave a sandwich of liquid sample between two slices of air, Figure 7. The volume of the liquid sample could be read accurately by means of the gradations of the barrel. After the needle was thrust through the septum, the plunger could be pushed all the way into the barrel. The air ahead of the sample permitted accurate measurement of the volume of contained liquid and the air behind the liquid permitted all of the sample to be injected into the chromatograph.

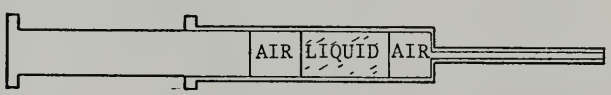


Fig. 7 - Method to Correct Injection Errors

General conditions for gas chromatographic analyses were as follows. Any variations on these general conditions are specified under experimental procedures.

Column temperature was kept at 250°C, and injector port and flame ionization detector were maintained at 270°C. Nitrogen gas flow rate was approximately 40 ml/minute. A 6 foot × 1/4 inch coiled glass column of 3 percent SE 30 on Gas Chrom Q was used for all analyses except those noted otherwise.

Coliform analyses were done using the Membrane Filter technique. Standard procedures, as outlined in the 13th edition of *Standard Methods for the Examination of Water and Wastewater*, were used on all analyses.

CHAPTER V

OPTIMIZATION OF THE EXTRACTION, CLEANUP, AND GAS CHROMATOGRAPHIC ANALYSIS OF COPROSTANOL

The procedure used for analysis of coprostanol to date was somewhat long and complicated. Therefore a series of studies were initiated on modification or elimination of some of the steps.

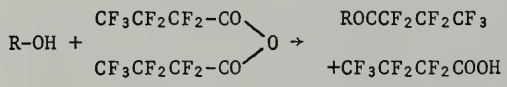
Either hexane or chloroform may be used to extract coprostanol from water samples.[5,6,7] It has long been recognized that chloroform will extract more non-lipid materials from sewage than will hexane.[87,88,89] Hexane is a more selective solvent than chloroform, since it will extract mostly non-polar material while chloroform also extracts many polar substances.

Acetonitrile, as well as dimethylformamide, was used in pesticide analyses to remove the chlorinated hydrocarbons from lipid-like material.[90,91] The lipid-like material remains in the hexane layer, while most pesticides, including the chlorinated hydrocarbons, are extracted into the acetonitrile or dimethylformamide. It may be possible to adapt the procedure for coprostanol by analyzing for the lipids in the hexane layer, and discarding the acetonitrile or dimethylformamide containing undesired substances.

There is disagreement over the necessity of preparing the trimethylsilyl (TMS) ether derivative of coprostanol prior to gas chromatography. Murtaugh and Bunch[5] prepared the TMS derivative, while others[6,7] found that the free sterol gave good results.

The limit of detection of coprostanol using a flame ionization detector is approximately 20 nanograms.[5] In real samples, this limit of detection id difficult to achieve because of interferences from other substances present in the water.

Picogram amounts of steroids have been analyzed by formation of the heptafluorobutyrate derivatives and detection using an electron capture (EC) detector.[92,93,94] The EC detector is extremely sensitive to electron-capturing groups such as fluorine atoms. The reaction is as follows:



If this reaction were to occur for coprostanol, the sensitivity for detection of coprostanol would be increased 1000-fold.

Some investigators have included a cleanup step employing thin-layer chromatography.[5,6,63,72] Murtaugh and Bunch[5] and Tabak *et al.*[72] claimed that TLC was necessary for all surface water samples regardless of concentration, while extracts from feces or wastewater plant effluents could be analyzed without TLC because of the relatively high concentration. Smith *et al.*[63] initially used a rather complicated liquid-liquid chromatrophic (LLC) procedure and later used TLC for cleanup of surface water as well as wastewater samples.[6]

Experimental

Gas Chromatography

Chromatography of free sterols

Several injections of free coprostanol, dissolved in hexane, were made. Column conditions were as described in Chapter IV. A typical chromatogram of coprostanol is shown in Figure 8.

Resolution of coprostanol and cholesterol

Chromatographs were obtained for coprostanol, cholesterol, and combined coprostanol and cholesterol under conditions described in Chapter IV. Cholestane was used as an internal standard to compare locations of peaks. Figure 9 shows that the coprostanol and cholesterol peaks overlap somewhat but the peak height for coprostanol is the true peak height when cholesterol is also present.

Electron Capture Detector

Procedure—experiment #1

To a small glass tube were added 0.5 µg coprostanol, 0.1 ml hexane, 2 µl of tetrahydrofuran, and 4 µl of heptofluorobutyric anhydride. The tube was stoppered and heated at 60°C for 30 minutes. The solvent was evaporated under nitrogen at about 40-50°C, and the dried residue was dissolved in 0.5 ml hexane. Microliter quantities were injected into a 3 percent SE 30 column for gas chromatographic analysis. Column conditions were as follows: inlet 250°C, column 230°C, detector 250°C. Ninety-five percent argon-5 percent methane was used as a carrier gas at a flow rate of approximately 120 ml/minute. Detection was with a Ni^{63} electron capture detector.

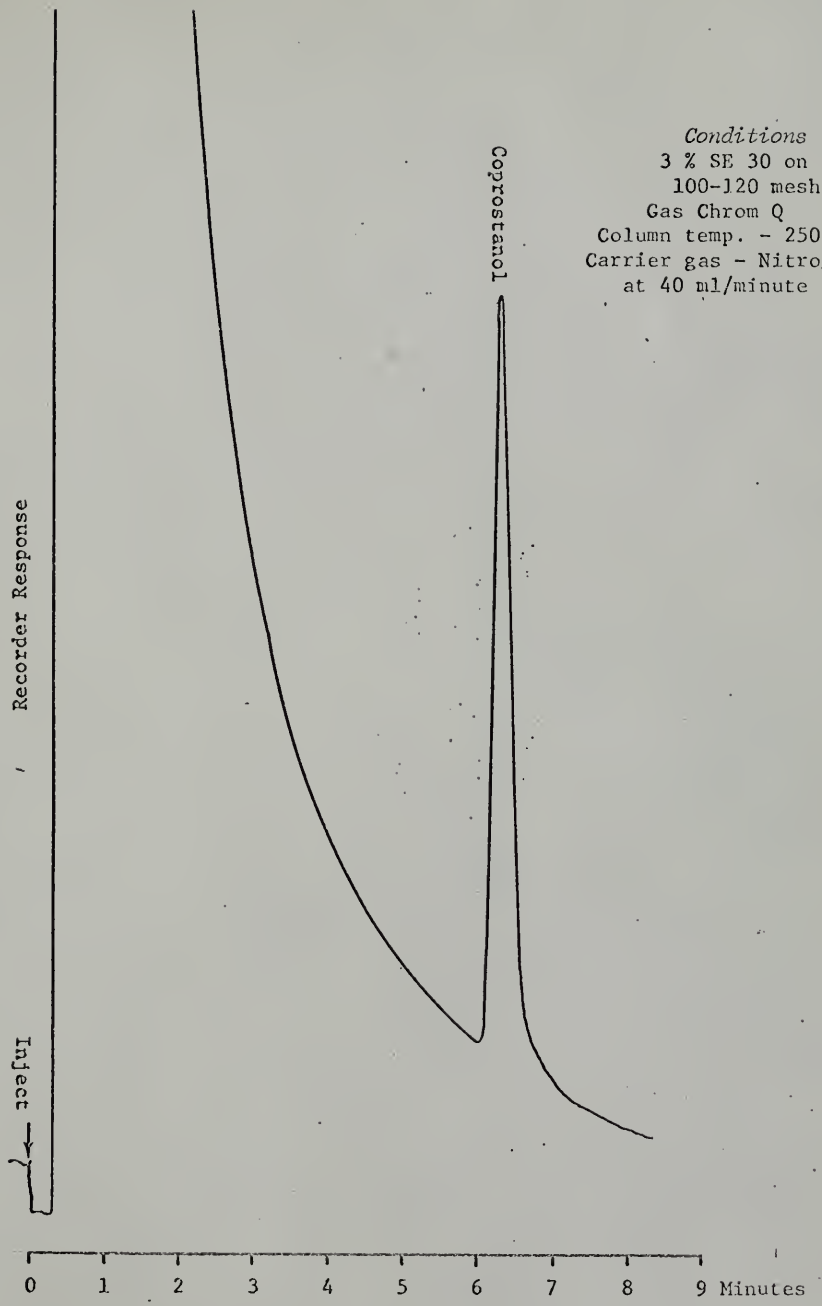


Fig. 8 - Chromatogram of Free Coprostanol

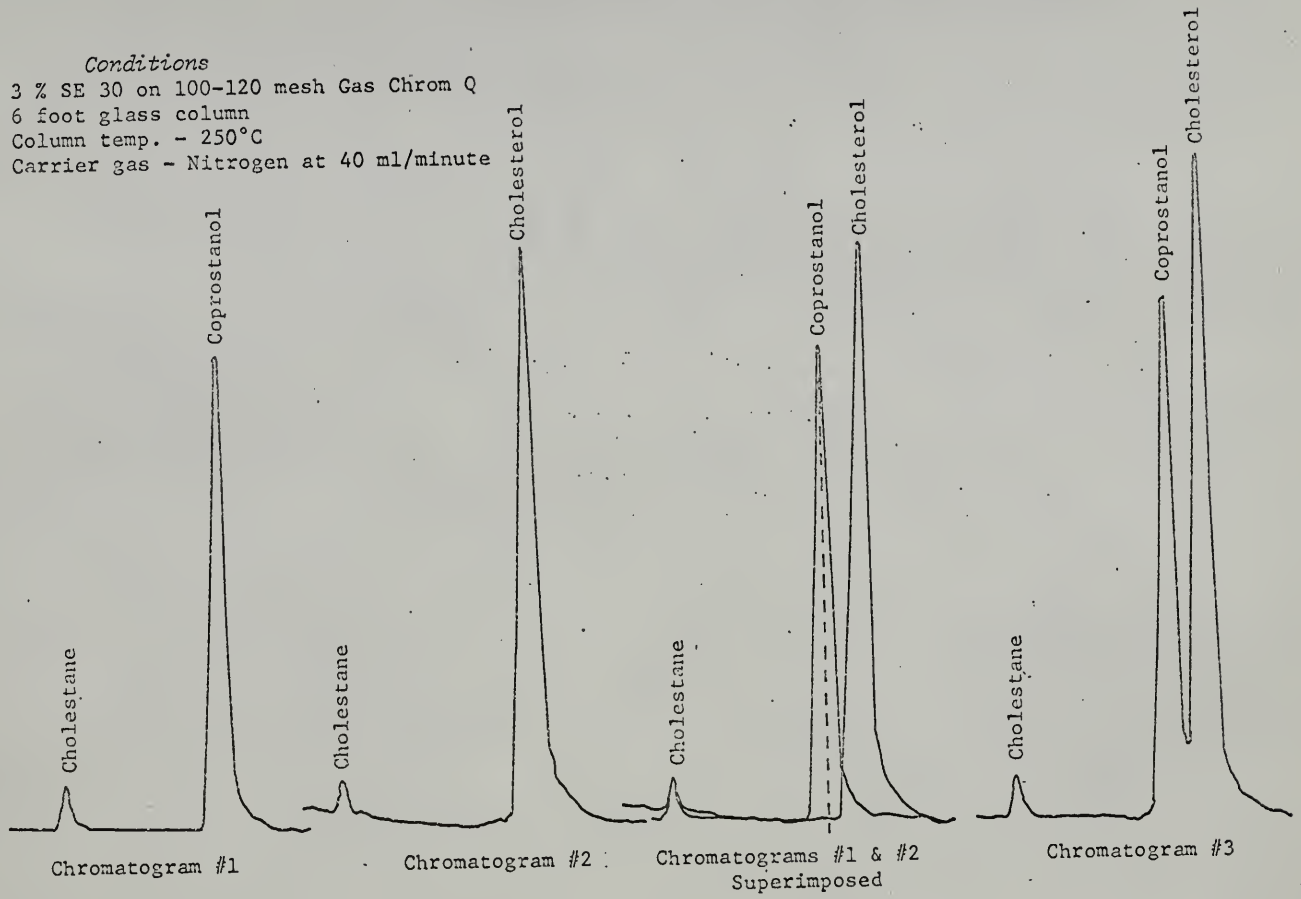


Fig. 9 - Chromatograms Showing Resolution of Cholesterol and Coprostanol

Procedure—experiment #2

To a small glass tube were added 5.1 µl coprostanol, 2 ml hexane, 5 µl tetrahydrofuran, and 10 µl heptafluorobutyric anhydride. The tube was then sealed shut with a flame and heated at 70°C for 30 minutes. Microliter samples were taken for gas chromatography. Conditions for gas chromatography were the same as for experiment #1.

Results of experiments #1 and #2

No peaks were obtained on any chromatograms for the heptafluorobutyrate derivative of coprostanol in either experiment.

Extraction and Cleanup

Procedure—experiment #1—cleanup with acetonitrile

Fifty µg of coprostanol was dissolved in one liter of distilled water, and the water was then extracted with two 50 ml portions of hexane (shaking for 2 minutes). The hexane extracts were combined and washed with two 25 ml portions of acetonitrile (shaking for 2 minutes). The acetonitrile washings were combined and reduced by flash evaporation to 1 ml. The hexane extracts were similarly reduced to 1 ml. Hexane extract, acetonitrile washings and standard coprostanol were run on a column of 3 percent QF 1 on Gas Chrom Q. Column temperature was 250°C and both inlet and flame ionization detector were at 270°C.

Results

Hexane extract

µl Injected	Peak Height
3.5	4.78 cm
3.5	4.55 cm
3.5	4.20 cm
	4.51 Avg.

Acetonitrile washings

µl Injected	Peak Height
3.5	0.14 cm

Reference coprostanol

µl Injected	Peak Height
3.5	4.66
3.5	4.54
	4.60 Avg.

% Recovery of coprostanol in hexane = $\frac{4.51}{4.60} \times 100 = 98.1\%$

% Recovery of coprostanol in acetonitrile = $\frac{0.14}{4.60} \times 100 = 3.04\%$

Total Recovery = 98.1 + 3.04 = 101.1%

Procedure—experiment #2—cleanup with 70 percent ethanol and acetonitrile

Forty µg coprostanol was added to one liter of distilled water. Two ml HCl (conc) and 5 ml 20% NaCl were added to the solution. The water sample was extracted with two-50 ml portions of hexane (shaking for 2 minutes) and then washed with two-25 ml portions of 70% ethanol, followed by two-25 ml portions of acetonitrile. The combined hexane extracts were evaporated to dryness and then redissolved in 0.5 ml benzene.

The above procedure was repeated on two more samples and GLC was run on the three extracts, using a column of 3 percent SE 30 on Gas Chrom Q. Column temperature was 250°C, inlet temperature 270°C, and flame ionization detector temperature was 270°C. A standard sample of coprostanol was prepared by dissolving 40 µg coprostanol in 0.5 ml benzene. Three-aliquot samples of the standard solution were run on GLC.

Results

Data are given in terms of peak height obtained per 1 µl injected onto the column from a total sample of 0.5 ml.

Peak Height/µl Injected Standard Solution	Peak Height/µl Injected Samples
3.93 cm/µl	3.63 cm/µl
4.06 cm/µl	3.82 cm/µl
3.74 cm/µl	3.63 cm/µl
3.91 cm/µl Avg.	3.69 cm/µl Avg.

$$\% \text{ Recovered} = \frac{3.69}{3.91} \times 100 = 94.4\%$$

The 95% C.I. can be calculated as $\mu = \bar{x} \pm t_{n-1}\ (\alpha/2)\ 5/\gamma_m$

$$= 94.4 \pm \frac{4.3(2.05)}{3}$$

$$= 94.4 \pm 5$$

Procedure—experiment #3—cleanup with N,N-dimethylformamide

Five hundred µg of coprostanol was added to 50 ml hexane and the hexane was washed with two-25 ml portions of N,N-dimethylformamide. The hexane was washed with one-25 ml portion of distilled water to remove all traces of N,N-dimethylformamide. The hexane was evaporated to dryness by flash evaporation and the dried residue was redissolved in 1 ml hexane. The procedure was repeated on another sample. A column of 3 percent SE 30 on Gas Chrom Q was used. Column temperature was 250°C and inlet and detector were 270°C.

Results

Peak Height/µl Injected Standard	Peak Height/µl Injected Sample #1	Peak Height/µl Injected Sample #2
2.19 cm/µl	0.754 cm/µl	0.753 cm/µl
2.55 cm/µl	0.705 cm/µl	0.748 cm/µl
2.24 cm/µl	0.808 cm/µl	0.810 cm/µl
2.09 cm/µl	0.756 cm/µl Avg.	0.770 cm/µl
2.31 cm/µl		
2.28 cm/µl Avg.		

% Recovery

$$\text{Sample \#1} = \frac{0.756}{2.28} \times 100 = 33.2\%$$

$$\text{Sample \#2} = \frac{0.770}{2.28} \times 100 = 33.8\%$$

Procedure—experiment #4—thin layer chromatography (TLC)

A number of environmental water samples were run to determine the necessity of TLC as a preliminary cleanup step. It was found that TLC was necessary for samples with concentrations below approximately 2 µg/liter, using 1-liter samples, but TLC may be omitted on samples with higher coprostanol concentrations. Figure 10 shows a chromatogram of an environmental sample with no prior cleanup by TLC while Figure 11 shows a chromatogram of a sample which required cleanup by TLC.

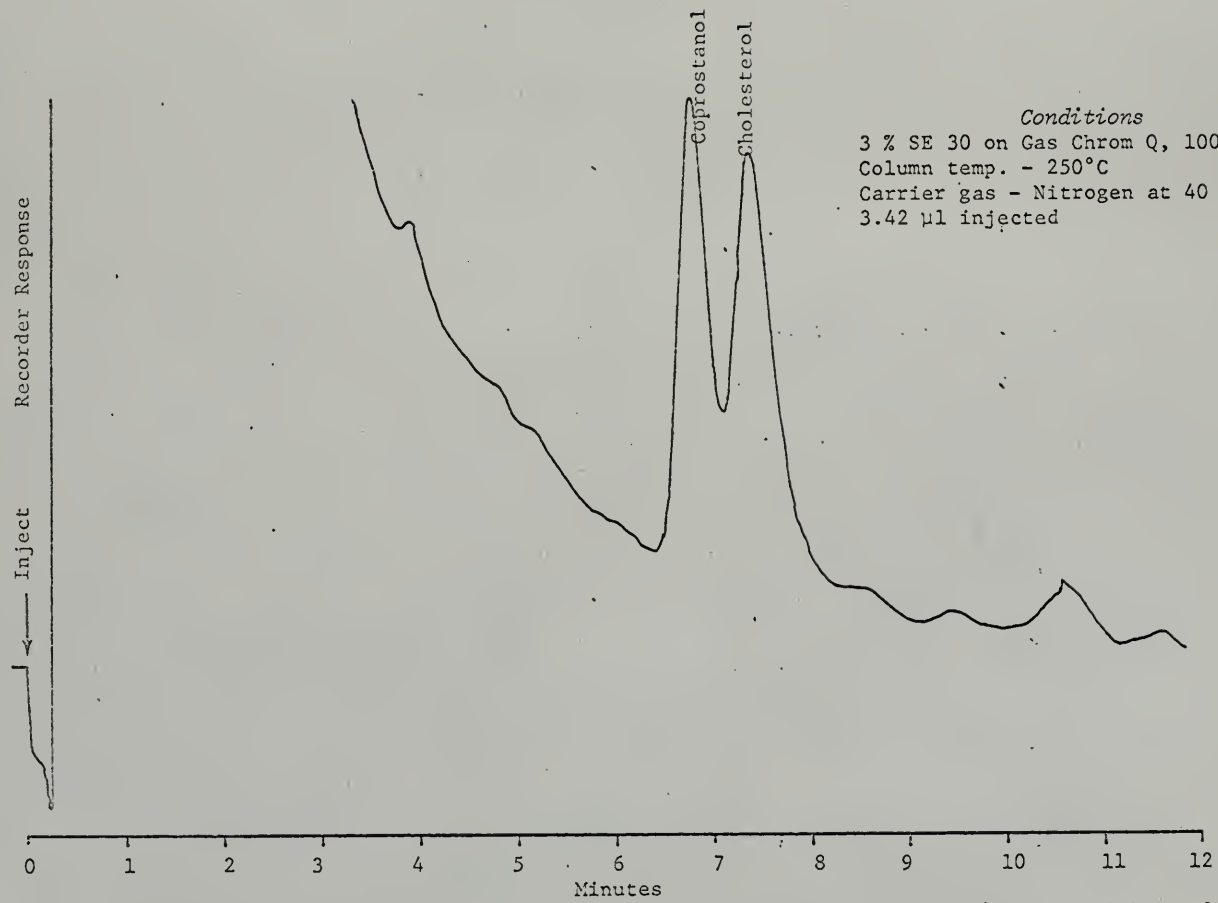


Fig. 10 - Environmental Sample - No Cleanup by Thin Layer Chromatography (Calculated Conc. 24.3 µg/l)

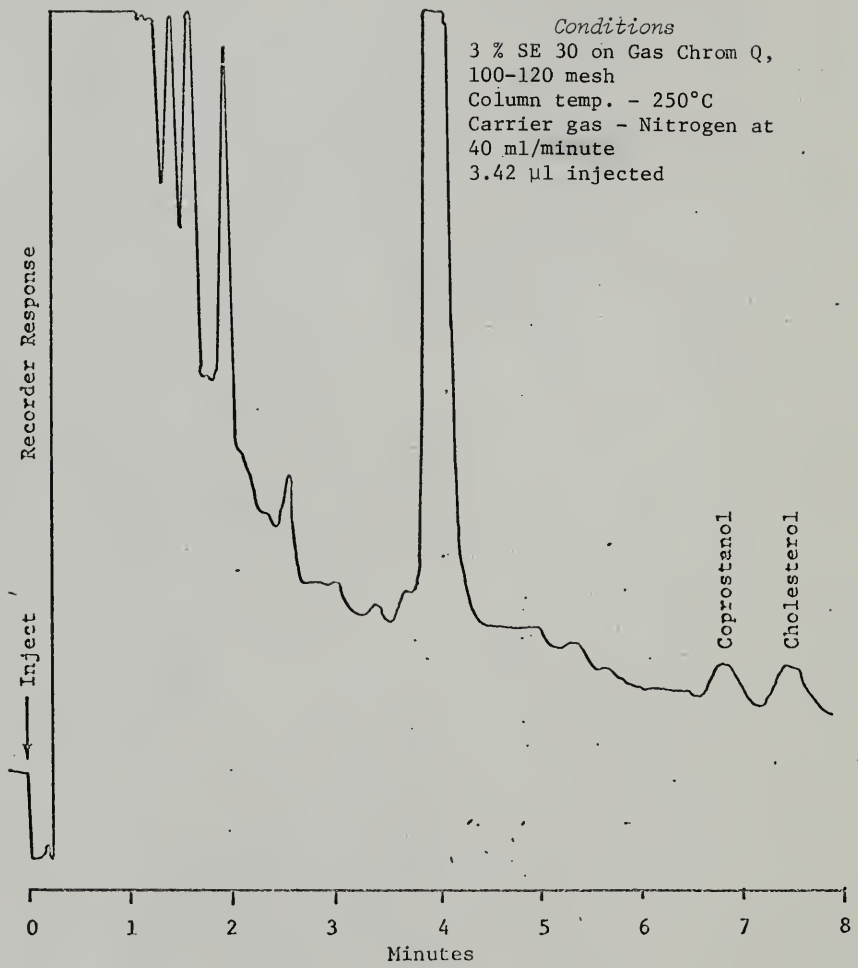


Fig. 11 - Environmental Sample - Cleanup by Thin Layer Chromatography (Calculated Conc. 0.22 µg/l)

CHAPTER VI

STUDIES ON THE SAPONIFICATION OF COPROSTANOL

No one has determined the quantitative difference between a procedure for coprostanol analysis including saponification compared to one omitting saponification. Saponification is a time-consuming step in the analytical scheme, requiring three hours out of a total analysis time of four hours. The saponification step could be omitted if the amount of coprostanol in the form of esters were small.

Experimental

Survey of Effect of Saponification on the Analysis of Wastewaters

Procedure—experiment #1

Samples were obtained from three wastewater treatment plants in the Gainesville, Florida area. The three plants sampled were the City Plant [2.7 million gallons per day (MGD), contact stabilization and 2.2 MGD, trickling filter], the University Plant (1.3-1.8 MGD, contact stabilization and 0.7 MGD, trickling filter), and the Sunland Training Center Plant (0.3 MGD, trickling filter).

Sample volumes varied from 125 ml to 1000 ml. Two samples were taken simultaneously from each location. A 500 ml and a 1000 ml sample were extracted with two 50 ml portions of hexane, and the combined hexane extracts were then washed with two 25 ml portions of 70 percent ethanol and one 25 ml portion of acetonitrile.

The hexane extracts were evaporated on a rotary evaporator using a 500 ml Kuderna-Danish evaporative concentrator with a 5 ml receiver. Results of these analyses are shown in Table 3. Figures 12 and 13 show typical chromatograms from samples before and after saponification.

Results

TABLE 3

Survey of Effect of Saponification on the Analysis of Wastewaters

	µg/l Coprostanol No Saponification	µg/l Coprostanol Saponified
City Influent	448	493
University Influent	340	412
Sunland Influent	755	741
City Effluent (Tricking Filter)	91.5	84.6
University Effluent (Trickling Filter)	72.6	70.0
Sunland Effluent (Trickling Filter)	181	168
City Effluent (Contact Stabilization)	16.1	9.1
University Effluent (Contact Stabilization)	1.8	1.0

Statistical analysis

$H_0 \equiv \mu\ \%_D = 0$

$H_1 \equiv \mu\ \%_D = 0$

$\alpha = 0.2$

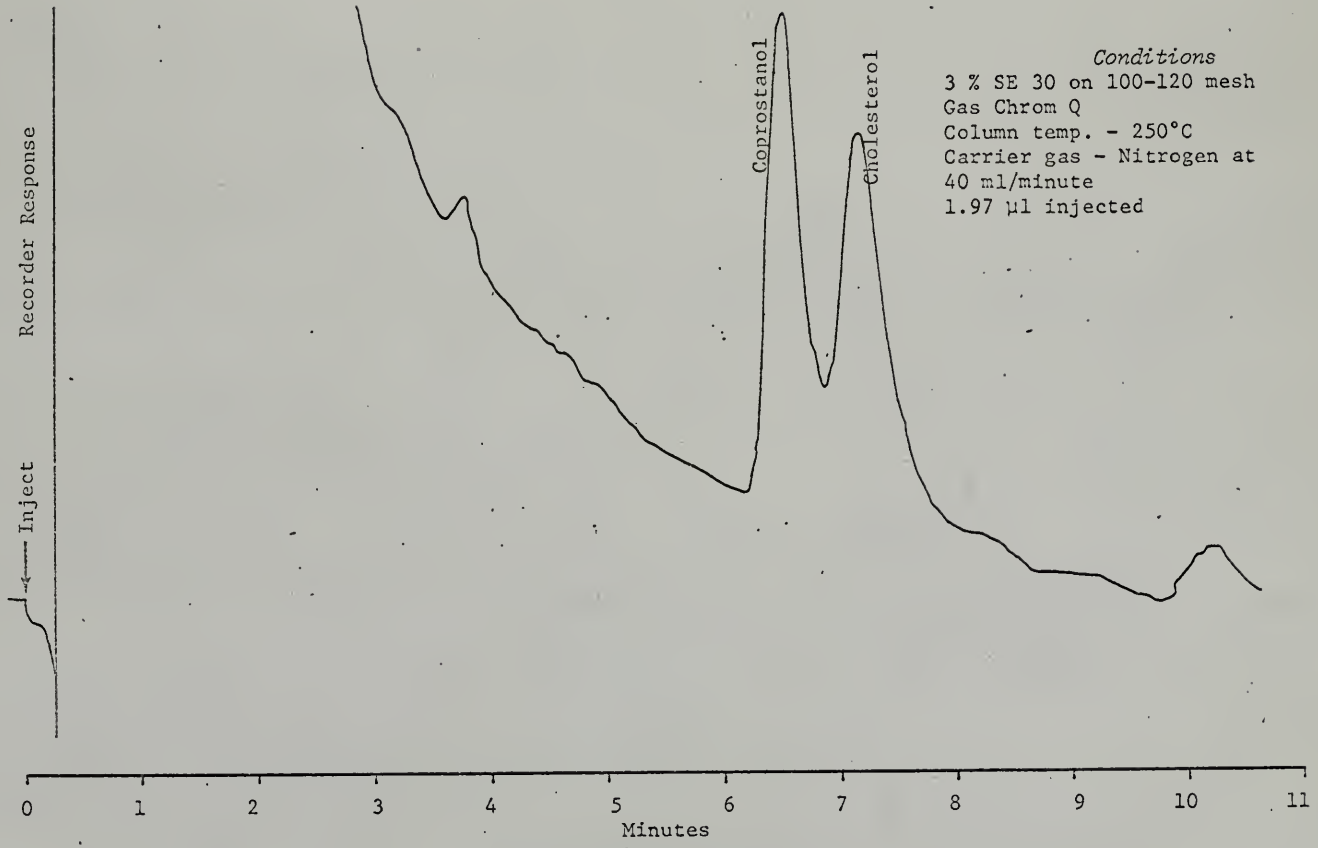


Fig. 12 - Chromatogram
Extract of Effluent from City of Gainesville Trickling Filter Plant Before Saponification

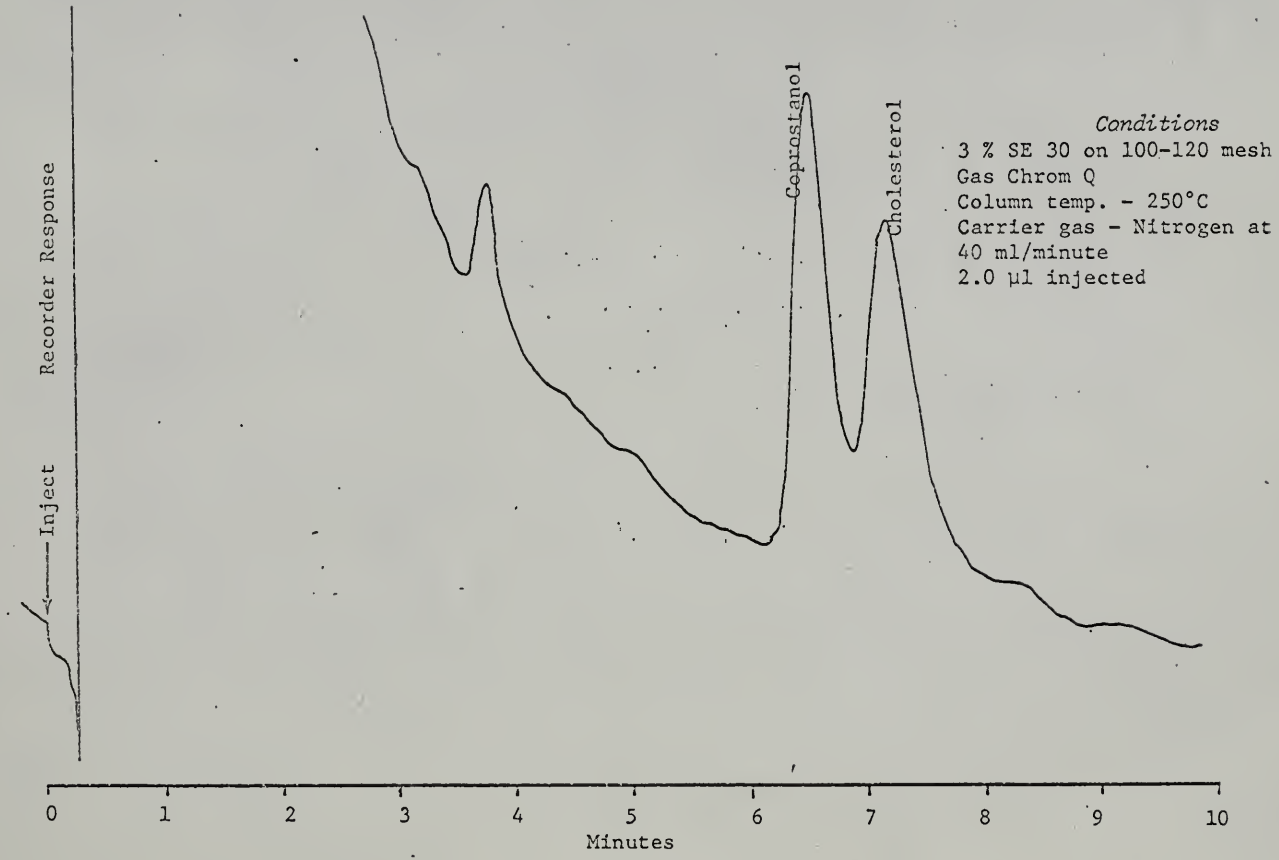


Fig. 13 - Chromatogram
Extract of Effluent from City of Gainesville Trickling Filter Plant After Saponification

Sample	No Sapon	Sapon	% D	(% D)^2
1	448	493	-10.04	100.8
2	340	412	-21.17	448.2
3	755	741	+ 1.85	3.42
4	91.5	84.6	+ 7.54	56.9
5	72.6	70.0	+ 3.58	12.8
6	181	168	+ 7.18	51.5
7	16.1	9.1	+43.47	1,890
8	1.8	1.0	+44.44	1,975
			+76.85	4,538.62

$\overline{\%\ D} = 9.6$

Therefore $\sigma_{\%D} = \frac{1}{7}\left[\Sigma(\%D)^2 - \frac{(\Sigma\%D)^2}{8}\right] = 542.9$

Then $t = \frac{\overline{\%D} - 0}{\sqrt{\sigma_{\%}^2/8}} = 1.16$

From standard statistical tables, two-sided $t_{20\%} = 1.415$

Not significant
H_0 accepted

Effect of Saponification on the Analysis of Effluent from Activated Sludge Process

Procedure—experiment #2

A 10-liter sample was taken from the effluent of the contact stabilization unit of the University Treatment Plant. This sample was divided into one-liter samples, each of which was preserved by the addition of 1 ml concentrated H_2SO_4. Five ml of 20 percent NaCl solution was added to each sample, and the samples were extracted in the usual manner with hexane. The hexane was reduced to dryness, the residue redissolved in 0.5 ml hexane, and chromatographed by GLC. The concentration of coprostanol in each sample was estimated from peak heights. The ten samples were then each dissolved in 50 ml of 7.5 percent KOH in 70 percent ethanol, and then refluxed for three hours.

After this saponification step, each sample was diluted with 50 ml distilled water, and coprostanol was re-extracted with two 25 ml portions of hexane. The hexane was washed with two 15 ml portions of 70 percent ethanol, evaporated to dryness, and the residue redissolved in 0.5 ml hexane. The concentration of coprostanol in these extracts was then determined from the peak heights after gas-liquid chromatography. Results of these analyses are given in Table 4. Figures 14 and 15 show typical chromatograms of contact stabilization effluent run before and after saponification.

TABLE 4

Effect of Saponification on the Analysis of Effluent from the Activated Sludge Process

Sample Number	µg/l Coprostanol Before Saponification	µg/l Coprostanol After Saponification
1	4.55	4.92
2	4.75	5.77
3	4.90	5.15
4	4.30	5.49
5	4.30	5.79
6	4.20	6.18
7	4.20	4.74
8	4.65	5.95
9	4.45	5.49
10	4.25	4.28

Statistical analysis

$$Ho = \frac{n\sigma 1^2}{\sigma_0^2} = \frac{\sigma_0^2}{\sigma_0^2}$$

Calculating

$$F = \frac{n\sigma 1^2}{\sigma_0^2} = 22.94$$

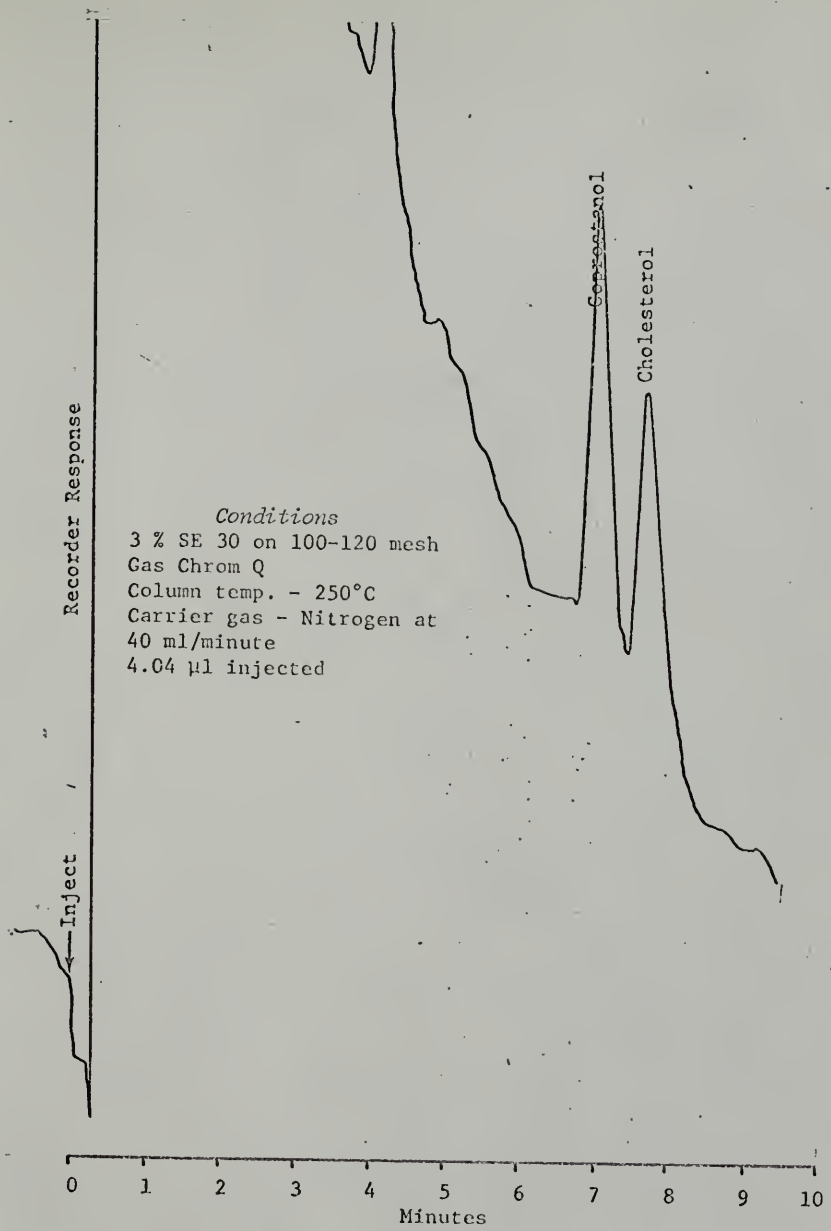


Fig. 14 - Chromatogram
Unsaponified Extract of Effluent from University of Florida
Activated Sludge Sewage Treatment Plant

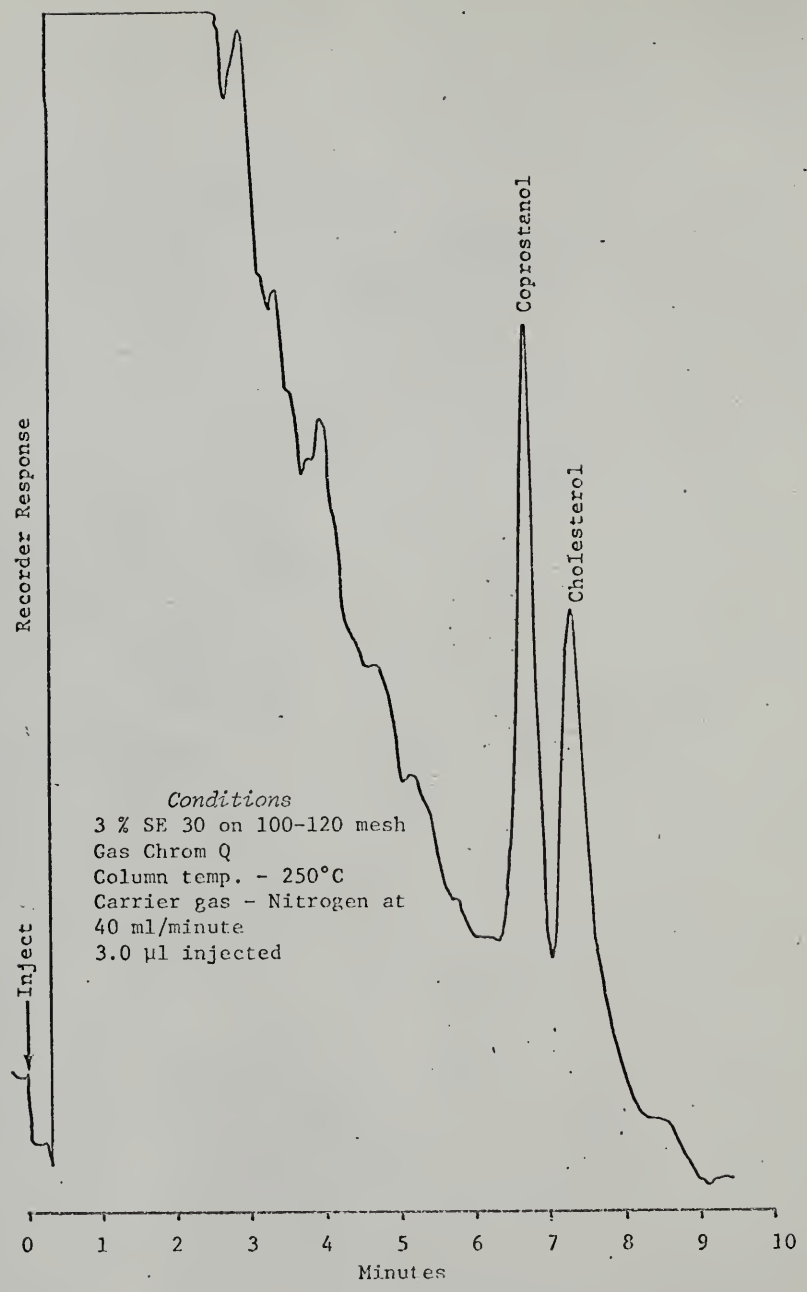


Fig. 15 - Chromatogram
Saponified Extract of Effluent from University of Florida Activated Sludge Sewage Treatment Plant

From standard statistical tables,

$F_{1\%} = 8.28$

Result is significant

Ho can be rejected and it can be concluded that there is a real difference between the two methods.

Calculating the 95 percent confidence interval for sample means we get:

Before saponification 4.45 ± 0.14

After saponification 5.33 ± 0.30

Effect of Saponification on the Analysis of Raw Sewage Influent

Procedure—experiment #3

The same procedure as in experiment #2 was used except that the source of the sample was raw influent from the University Sewage Treatment Plant. The five-liter sample was divided into ten samples of 500 ml each. Results of these analyses are given in Table 5. Figures 16 and 17 show typical chromatographs of raw sewage extracts before and after saponification.

TABLE 5

Effect of Saponification on the Analysis of Raw Sewage Influent

Sample Number	µg/l Coprostanol Before Saponification	µg/l Coprostanol After Saponification
1	554	600
2	522	551
3	576	*
4	520	584
5	548	590

TABLE 5—*Continued*

Sample Number	µg/l Coprostanol Before Saponification	µg/l Coprostanol After Saponification
6	536	599
7	522	585
8	504	549
9	502	576
10	492	560

*Sample was lost.

Statistical analysis

$$Ho \equiv \frac{n\sigma^2}{\sigma_0^2} = \frac{\sigma_0^2}{\sigma_0^2}$$

Calculating

$$F = \frac{nl^2}{\sigma_0^2} = 21.52$$

From standard statistical tables,

$$F_{1\%} = 8.40$$

Result is significant, therefore Ho can be rejected and it can be concluded that there is a real difference between the two methods. The 95 percent confidence interval can be calculated

Before sapon = 528 ± 15

After sapon = 577 ± 12

Effect of Saponification on the Analysis of Trickling Filter Effluent

Procedure—experiment #4

A ten-liter sample was taken from the trickling filter effluent of the University Sewage Treatment Plant, and then divided

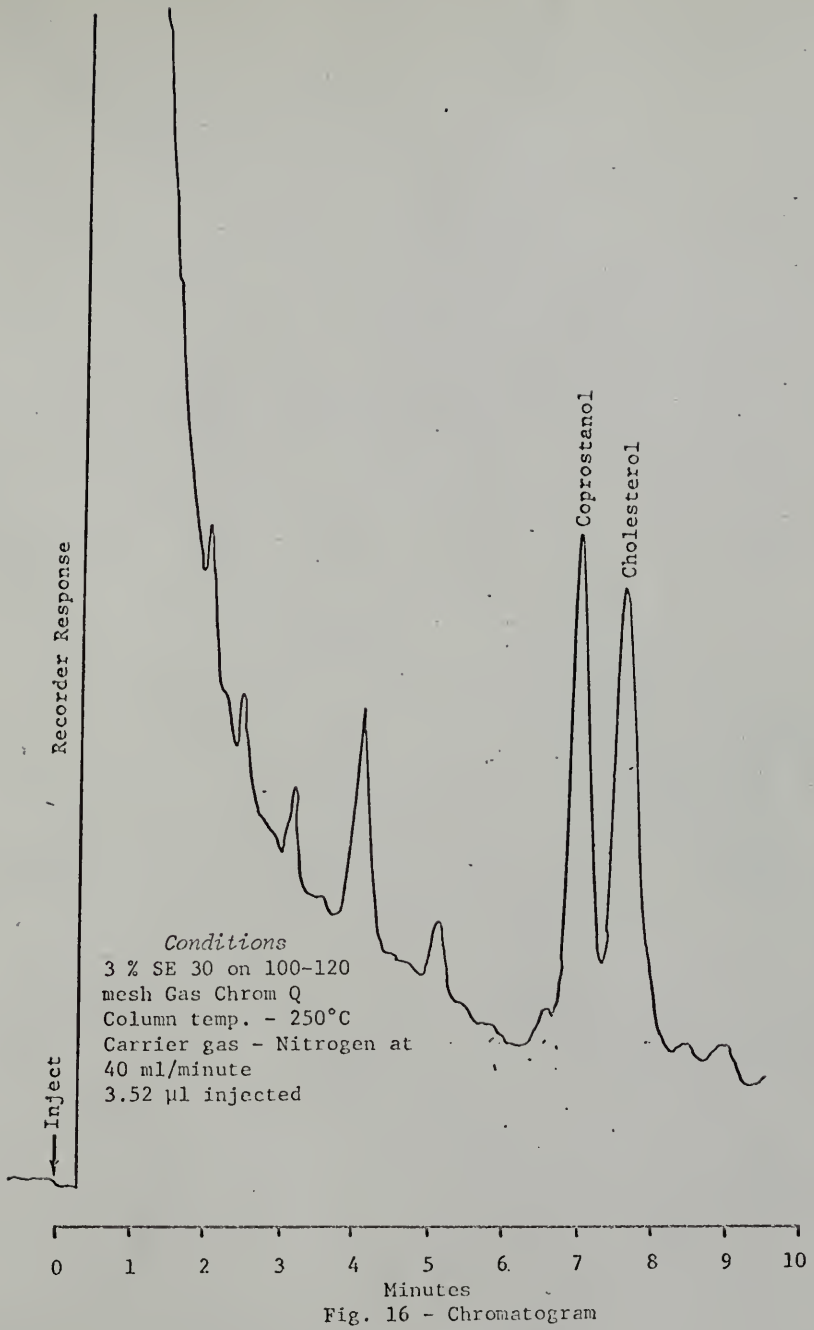


Fig. 16 - Chromatogram
Unsaponified Extract of Influent to University of Florida Sewage Treatment Plant

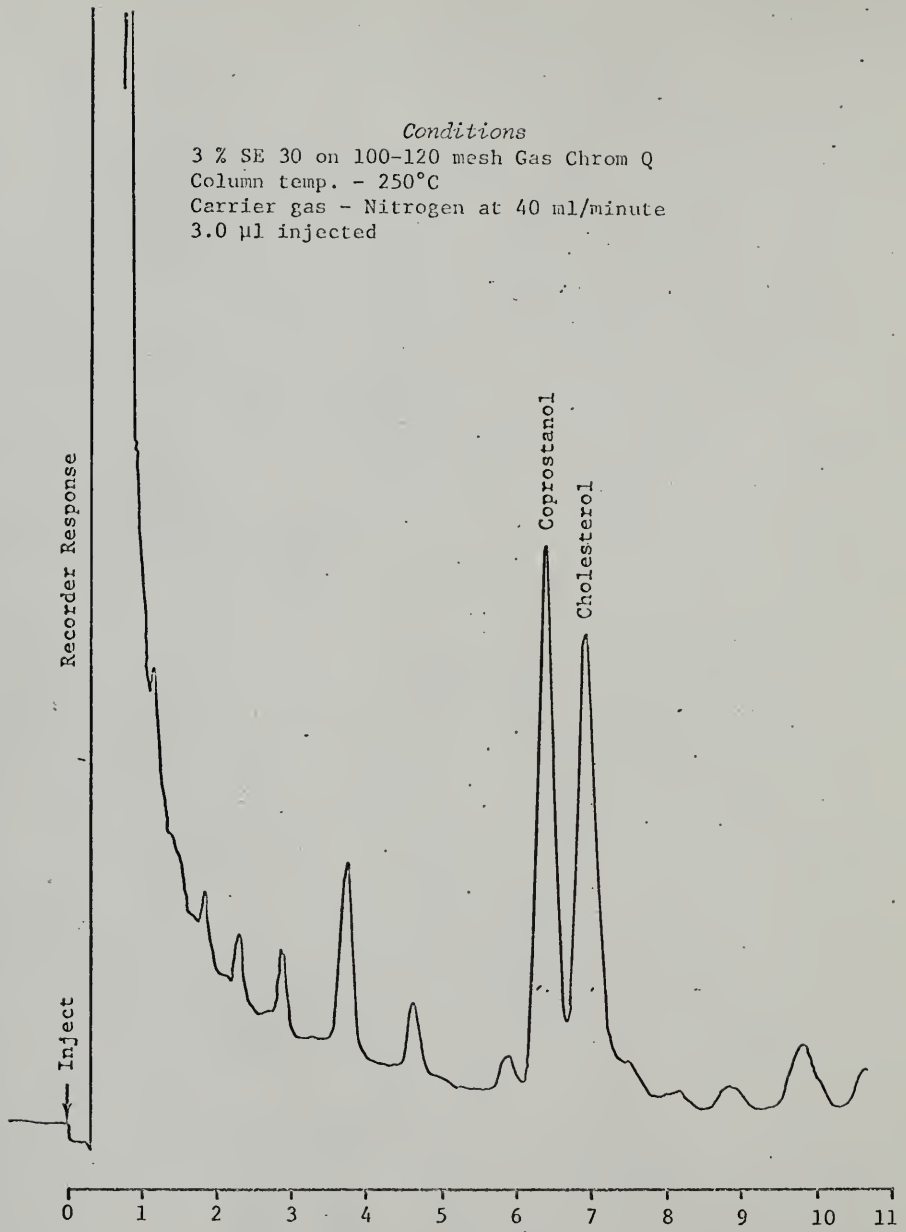


Fig. 17 -- Chromatogram
Saponified Extract of Influent to University of Florida Sewage Treatment Plant

into 20 samples of 0.5 liter each. Each sample was preserved with 1 ml of concentrated H_2SO_4 and 3 ml 20 percent NaCl was added.

Ten samples were extracted in the usual manner. They were not subjected to saponification. After normal extraction the other ten samples were saponified for three hours in 50 ml of 7.5 percent KOH in 70 percent ethanol. The saponified alcohol solutions were diluted with 50 ml of water, followed by a re-extraction with two 25 ml portions of hexane, and washed with two 15 ml portions of 70 percent ethanol.

Both unsaponified and saponified samples were analyzed by GLC. Results of these analyses are given in Table 6.

Figures 18 and 19 show typical chromatograms of trickling filter effluent before and after saponification.

TABLE 6

Effect of Saponification on the Analysis of Trickling Filter Effluent

Sample Number	µg/l Coprostanol Before Saponification	µg/l Coprostanol After Saponification
1	67.3	67.6
2	65.7	77.8
3	66.9	76.5
4	72.2	82.8
5	84.3	85.9
6	59.9	71.8
7	64.7	82.2
8	69.2	75.9
9	70.2	77.1
10	70.2	67.0
Average	69.1	76.4

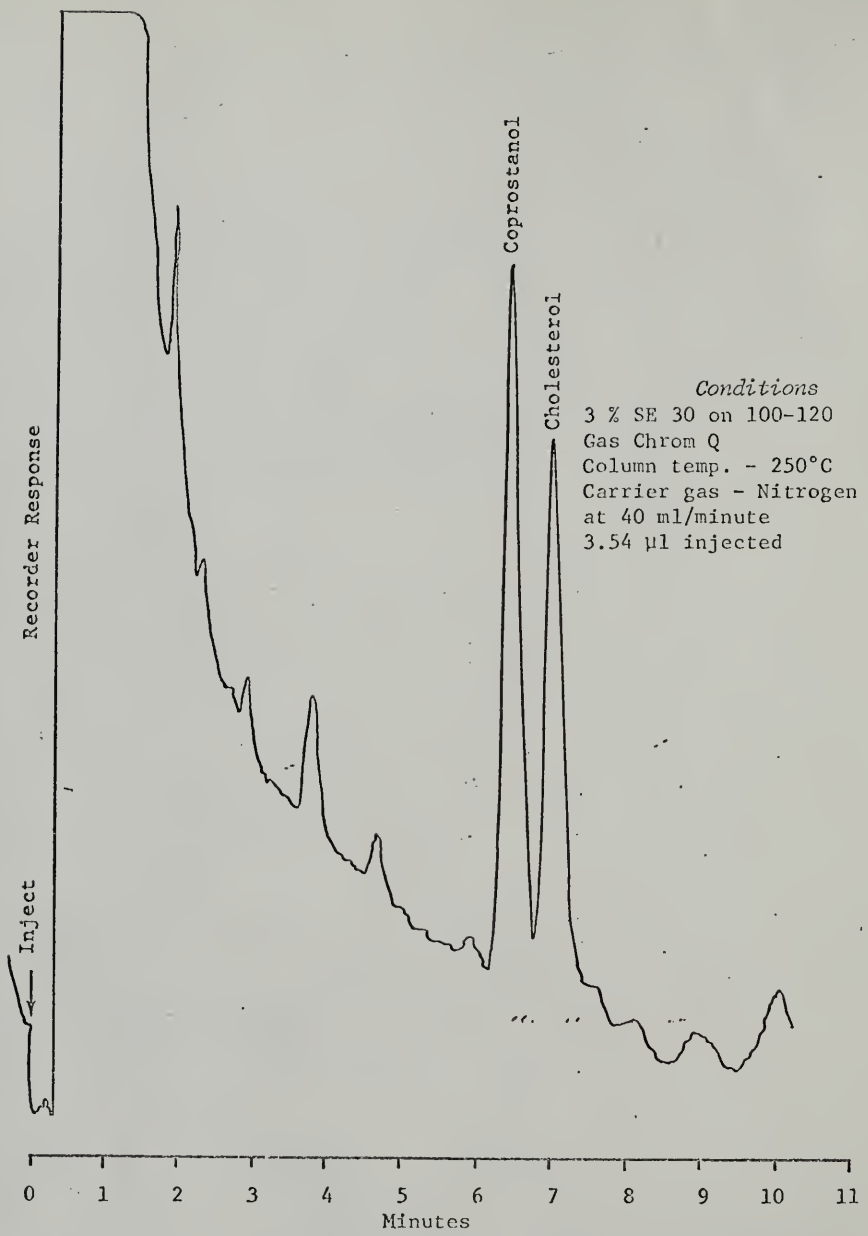


Fig. 18 - Chromatogram
Unsaponified Extract of University of Florida Trickling Filter Effluent

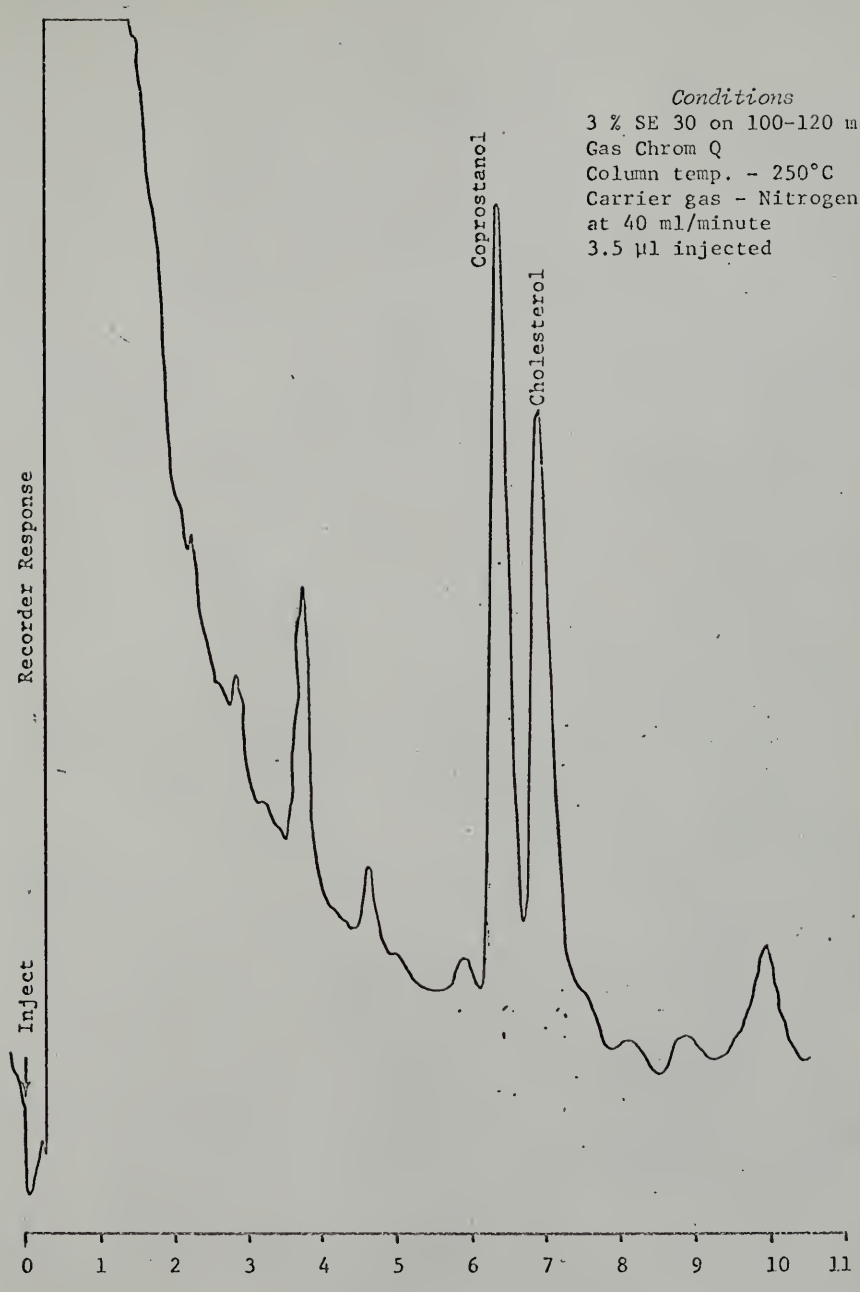


Fig. 19 - Chromatogram
Saponified Extract of University of Florida Trickling Filter Effluent

Statistical analysis

$$F \text{ calc} = \frac{n\sigma_1^2}{\sigma_0^2} = 5.97$$

$$F_{5\%}(1,18) = 4.41$$

$$F_{1\%}(1,18) = 8.28$$

Thus Ho can be rejected at the 5 percent value for α. The 95 percent confidence intervals can be calculated

After saponification 76.40 ± 1.54

Before saponification 69.06 ± 1.46

Precision of analyses

For those experiments in which several samples were taken for analyses, the precision of the analyses can be calculated.

	Average Error (Expressed as Percent of Mean)	Standard Deviation (Expressed as Percent of Mean)
Raw Sewage		
Before saponification	3.94	4.96
After saponification	2.79	3.40
Trickling Filter		
Before saponification	6.02	9.23
After saponification	6.18	9.93
Activated Sludge		
Before saponification	4.61	4.45
After saponification	9.23	11.2

CHAPTER VII

STABILITY OF COPROSTANOL TOWARD MICROBIAL DEGRADATION

It has been shown that species of Proactinomyces present in soils are capable of utilizing coprostanol as their sole source of carbon for growth.[74] In addition more than 1,000 bacterial strains are capable of degrading cholesterol which is structurally almost identical to coprostanol.[84]

No experiments have been reported showing that the disappearance of coprostanol in sewage treatment is caused by biodegradation rather than physical removal into sludge, sediments, etc. If the disappearance were due to biodegradation, then the rate of disappearance should be affected by chlorination of sewage effluent. In addition, the rate of disappearance of coprostanol is of interest in determining the usefulness of coprostanol as an indicator of fecal pollution.

Smith and Gouron have stated that the removal of coprostanol by the activated sludge process may be due to a physical removal by the floc rather than a biodegradation.[6]

Laboratory experiments were performed to determine the rate of disappearance of coprostanol in both chlorinated and unchlorinated sewage effluents. At the same time microbiological analyses were made to see if the disappearance of coprostanol could be correlated with the decrease in indicator organisms. An experiment of exploratory

nature was also performed to determine the mechanism of coprostanol removal in the activated sludge process. Experiments were performed to determine the necessity of preserving samples.

Experimental

Degradation of Coprostanol

Procedure

Four samples of approximately 20 liters each were taken. Samples 1 and 2 were trickling effluent taken from the University Sewage Treatment Plant before chlorination. The third sample was chlorinated effluent from the University Plant and the fourth sample was a 1:2 mixture of chlorinated sewage effluent from the University Plant and a surface water taken from a small creek entering Payne's Prairie. All samples were taken to the laboratory and placed in large glass cylinders open to the atmosphere, where they were held and stirred constantly during the experiment.

Analyses for coprostanol were made on all samples over a number of days. Simultaneously with coprostanol analyses on samples 2, 3, and 4 total coliform, fecal coliform, and standard plate counts were taken. Microbiological tests were also made on the creek water before mixing with sewage effluent. In order to determine if there was any physical removal of coprostanol due to adsorption and deposition onto the glass cylinders, they were washed down with 50-75 ml hexane after termination of the experiments. The hexane was evaporated down to approximately 5 ml and aliquots were analyzed gas chromatographically. Results of the analyses of samples 1-4 are given in Tables 7-10.

Results

TABLE 7

Data for Sample #1—Trickling Filter Effluent Before Chlorination
(Sample Taken January 26, 1971)

Time, Hours	Coprostanol, µg/liter
0	95.5
3	78.7
6	86.8
12	76.7
24	47.9
48	21.8
100	11.4
144	5.71
216	5.38
244	4.0

No coprostanol was detected in the hexane washings of the glass cylinder after termination of the experiment. Figures 20, 21, and 22 show chromatograms run on identical size extracts (0.5 ml) and approximately identical syringe volumes for injection. The chromatograms correspond to 0 hours time, 48 hours time, and 144 hours time, or 95.5 µg/liter, 21.8 µg/liter, and 5.71 µg/liter respectively. Figure 23 shows a plot of the data for change in coprostanol concentration with time found in samples #1 and #2.

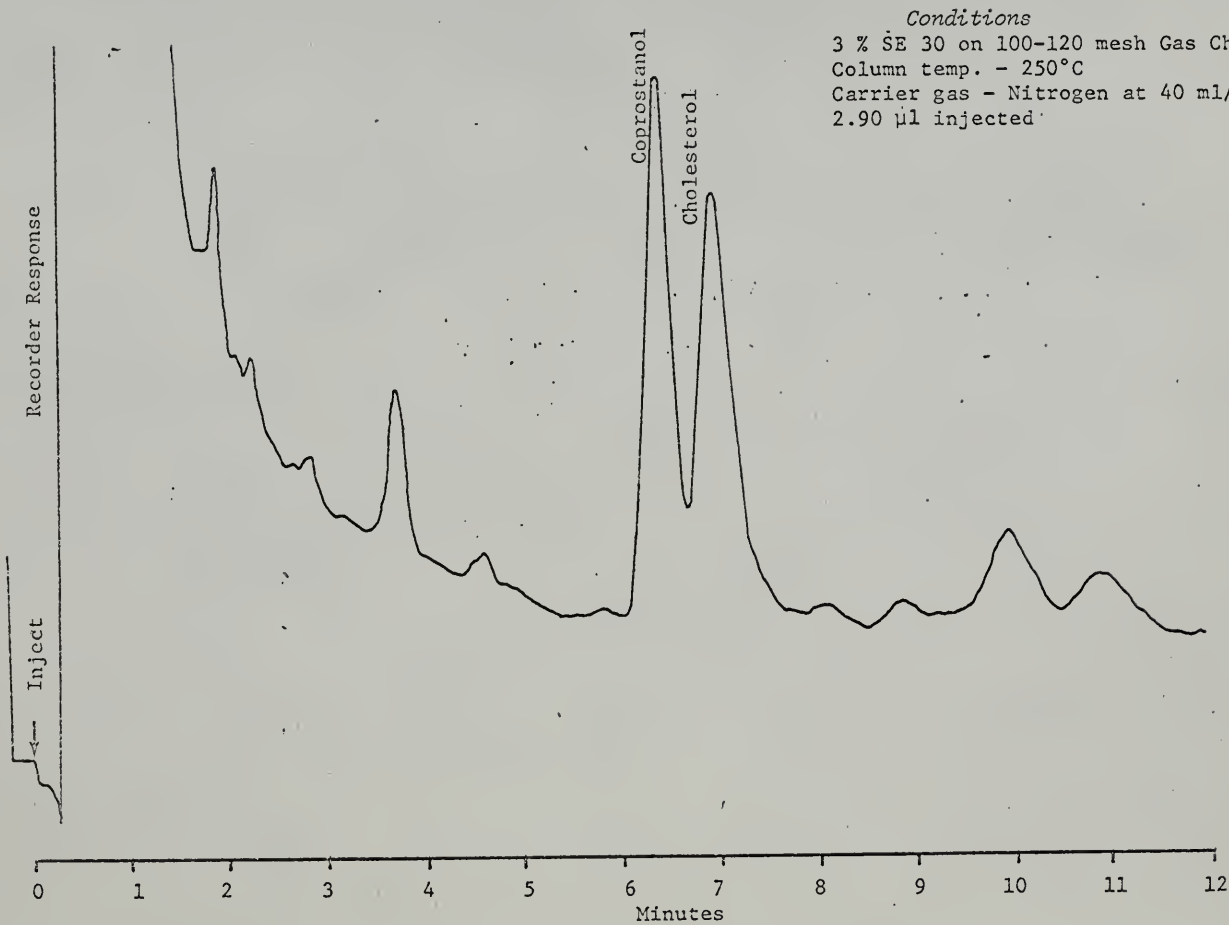


Fig. 20 - Chromatogram

Chromatogram of Sample #1 in Biodegradation Study After 0 Hours—(95.5 µg/liter coprostanol)

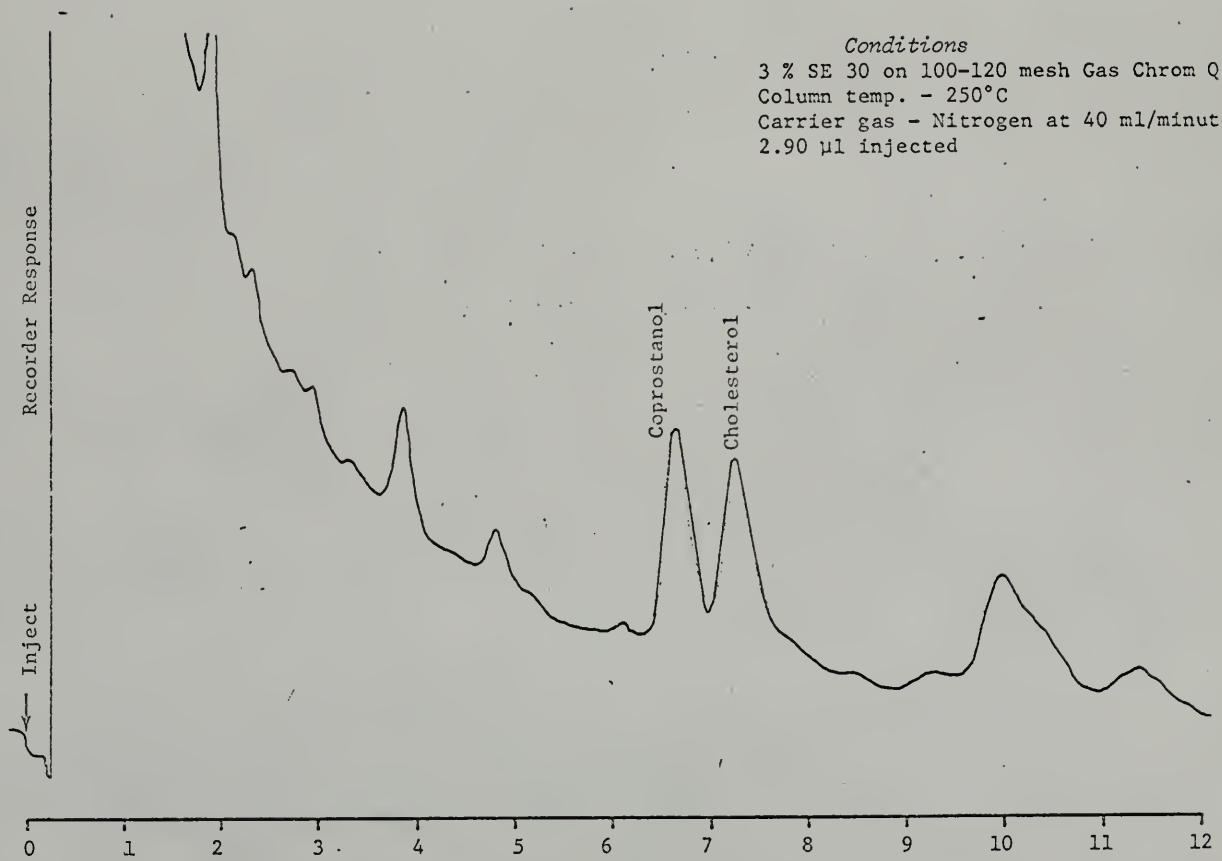


Fig. 21 - Chromatogram
Chromatogram of Sample #1 in Biodegradation Study After 24 Hours—(47.9 µg/liter coprostanol)

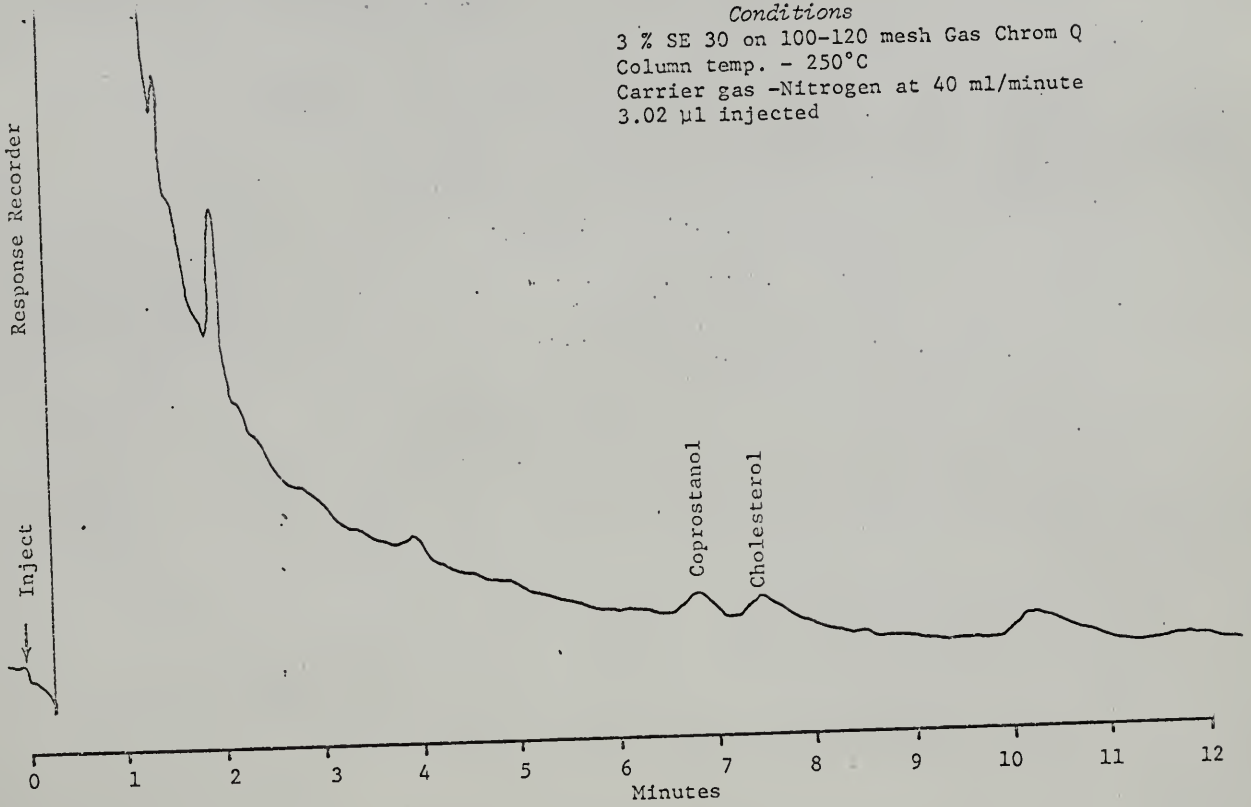


Fig. 22 - Chromatogram
Chromatogram of Sample #1 in Biodegradation Study After 144 Hours—(5.71 µg/liter coprostanol)

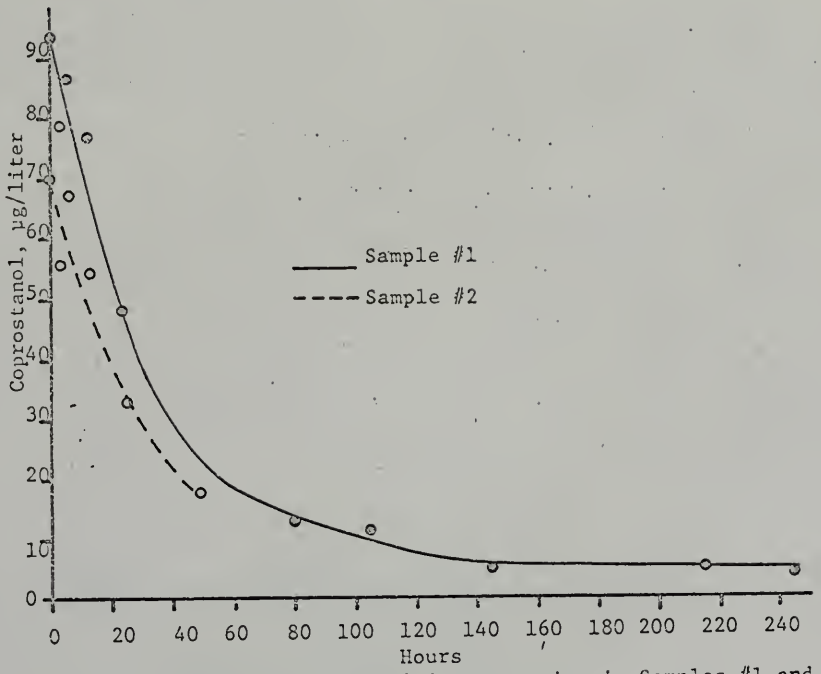


Fig. 23 - Rate of Change of Coprostanol Concentration in Samples #1 and #2 (Trickling Filter Before Chlorination)

TABLE 8

Data for Sample #2—Trickling Filter Effluent Before Chlorination
(Sample Taken February 23, 1971)

Time, Hours	Coprostanol µg/liter	Total Coliform Number/100 ml	Fecal Coliform Number/100 ml	Standard Plate Count Number/100 ml
0	70.2	9.7×10^6	4.2×10^5	7.4×10^8
3	55.8	4.7×10^6	2.9×10^5	1.04×10^9
6	67.8	-	-	-
12	54.2	-	-	-
24	32.7	4.2×10^5	3.4×10^4	7.3×10^7
48	17.6	4.8×10^5	2.95×10^4	4.56×10^7
96	-	6.2×10^5	1.41×10^4	5.8×10^7
192	-	4.1×10^5	5.9×10^3	2.15×10^7

No coprostanol was detected in the hexane washings of the glass cylinder after termination of the experiment.

TABLE 9

Data for Sample #3—University Sewage Plant Effluent After Chlorination
(Sample Taken February 23, 1971)

Time, Hours	Coprostanol µg/liter	Total Coliform Number/100 ml	Fecal Coliform Number/100 ml	Standard Plate Count Number/100 ml
0	42.6	3.2×10^2	2	1.4×10^4
3	39.4	1.7×10^3	9	2.04×10^6
6	41.1	-	-	-
12	40.9	-	-	-
24	47.1	2.08×10^4	0	5.9×10^5
48	35.2	6.6×10^3	2	TNC($>3\times10^6$)*
96	41.7	TNC($>3\times10^6$)	270	1.5×10^8
192	29.7	2.9×10^7	684	2.9×10^8

No coprostanol was detected in the hexane washings of the glass cylinder after termination of the experiment.

*TNC - Too Numerous to Count

TABLE 10

Data for Sample #4—University Sewage Effluent and Surface Water
(Sample Taken February 23, 1971)

Time, Hours	Coprostanol µg/liter	Total Coliform Number/100 ml	Fecal Coliform Number/100 ml	Standard Plate Count Number/100 ml
0	17.0	1.38×10^4	10	-
3	17.8	2.12×10^5	1300	8×10^5
6	15.0	-	-	-
12	15.7	-	-	-
24	12.2	2.12×10^4	-	1.5×10^6
48	13.9	3.8×10^5	36	TNC($>3\times10^6$)
96	17.0	2.3×10^7	-	8.9×10^8
192	7.7	3.4×10^6	2	3.5×10^7

Creek Water -	Total Coliform	Fecal Coliform	Standard Plate Count
	1.55×10^3	10	49×10^3

No coprostanol was detected in the hexane washings of the glass cylinder after termination of the experiment.

Figure 24 shows plots of the rate of change of coprostanol with time as found in samples 3 and 4. Figures 25, 26, and 27 show the rate of change of microbiological indicators with time as found in samples 2-4.

Activated Sludge

Procedure

One liter of return activated sludge from the University of Florida sewage treatment plant was taken and extracted with two 75 ml portions of hexane. It was found necessary to add approximately 100 ml 95 percent ethanol to break the emulsion that formed. The hexane was separated, washed with two 25 ml portions of ethanol and a gas chromatographic analysis was run on the hexane after reducing the volume to 2 ml by flash evaporation.

Results

A very complicated chromatograms was obtained with evidence of coprostanol, as well as cholesterol (see Figure 28). Large amounts of cholesterol were found to be present, with lesser amounts of coprostanol. No quantitative value for the amount of coprostanol could be determined due to the overlap of another, unknown peak, but approximately 100 µg/liter of coprostanol was present. This value was considerably less than the concentration found in incoming sewage (approximately 500 µg/liter) but more than that found in effluent from the activated sludge process (approximately 10 µg/liter). Thus it appears that a combination of physical removal and biological degradation may account for the removal of coprostanol, since physical removal would require that coprostanol concentration be higher in the concentrated biological floc than in the sewage influent, while complete biological

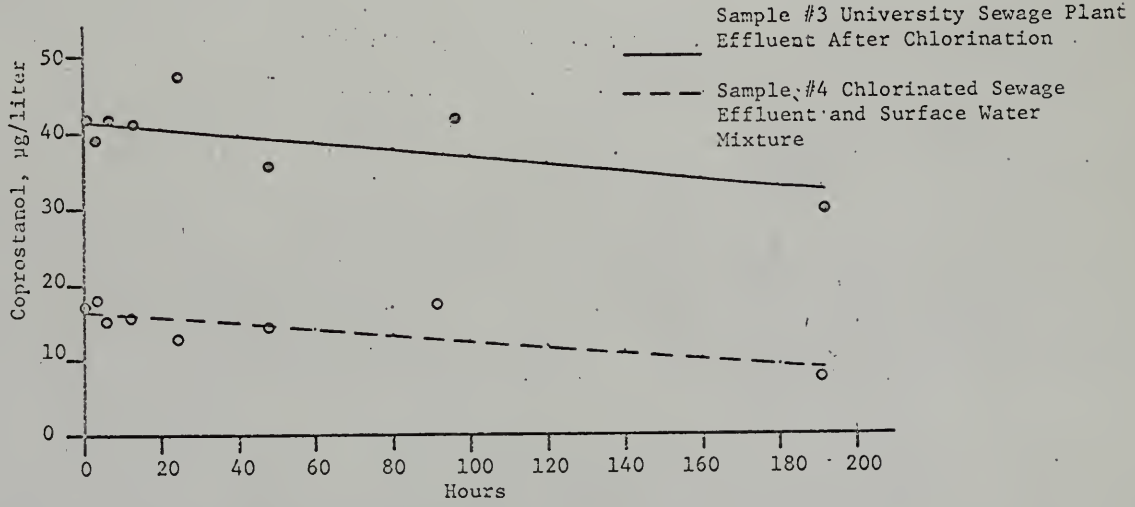


Fig. 24 - Rate of Change of Coprostanol Concentration in Samples #3 and #4

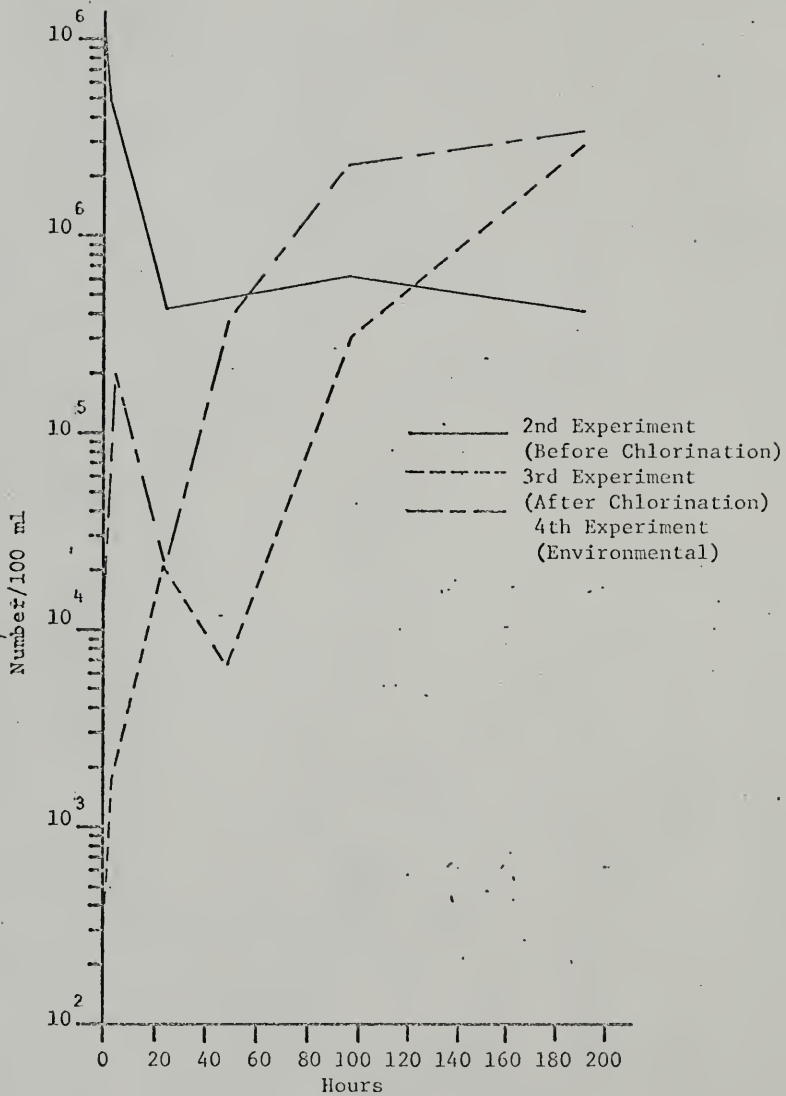


Fig. 25 - Total Coliform -- Rate of Change in Concentration

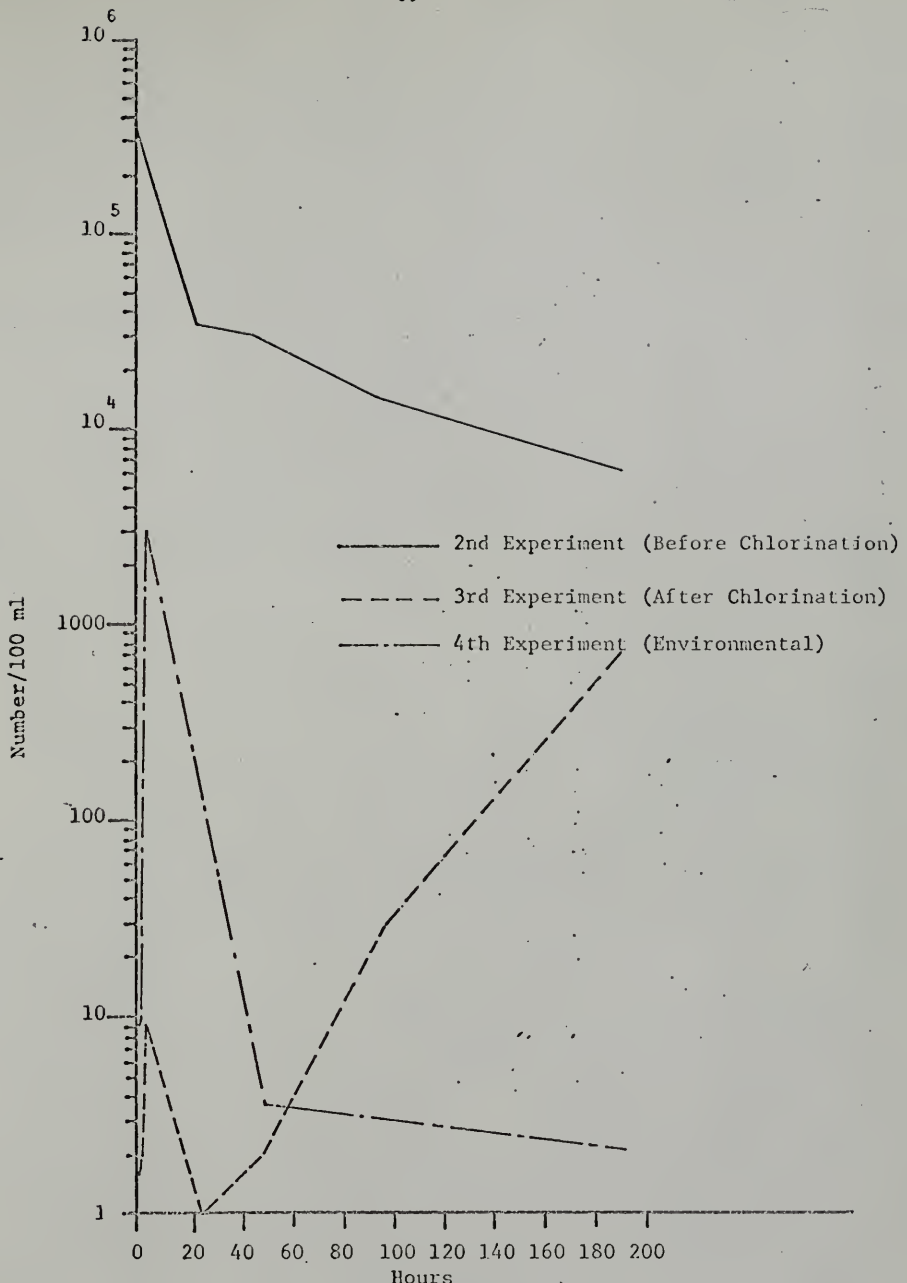


Fig. 26 - Fecal Coliform - Rate of Change in Concentration

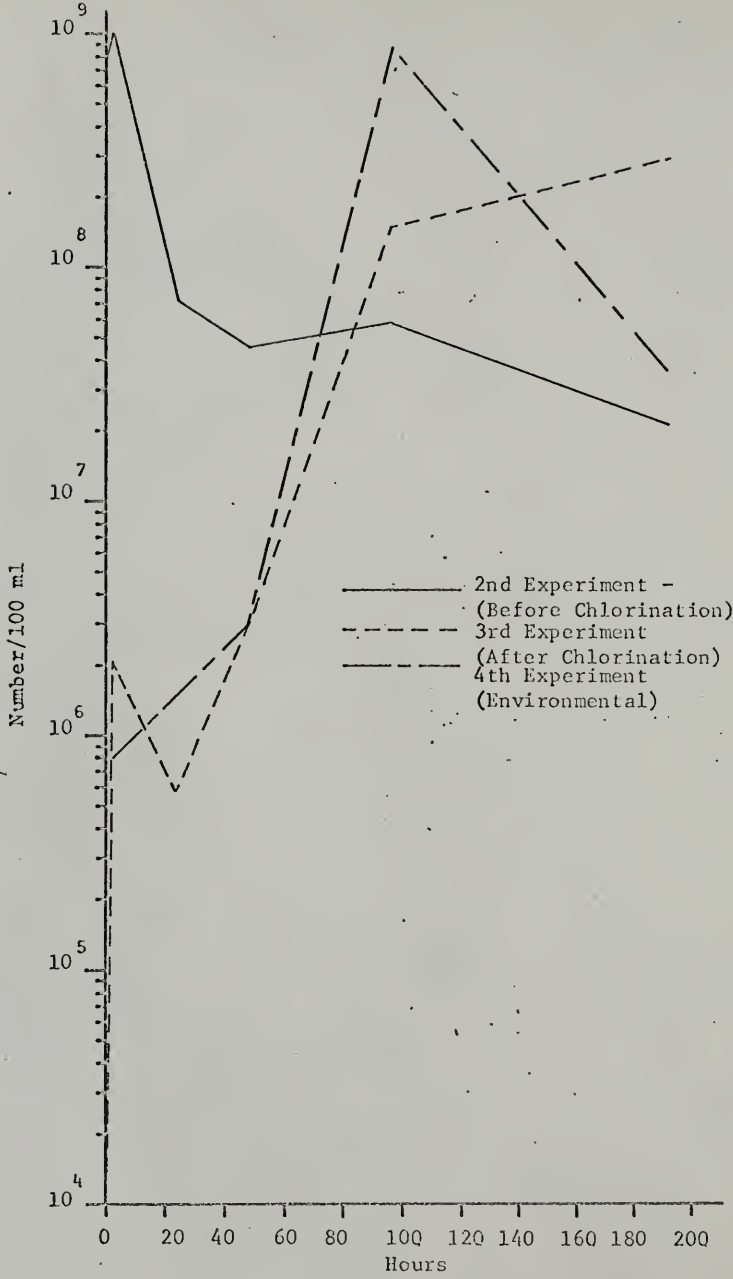


Fig. 27 - 48 hour Total Plate Count
Rate of Change in Concentration

Fig. 28 - Chromatogram of Extract of Return Activated Sludge

degradation would require that coprostanol concentration in the biological floc be no higher than that in the effluent.

Preservation of samples

Procedure

A 4 liter sample of trickling filter effluent was taken from the Gainesville Sewage Treatment Plant. The sample was divided into eight 500 ml samples. Two samples were preserved with 0.5 ml H_2SO_4 each and analyzed immediately upon return to the laboratory. Two samples were preserved by refrigerating at 4°C and analyzed after 8 days. Two samples were preserved with 0.5 ml H_2SO_4 each and preserved at room temperature. They were analyzed after 8 days in storage. Two samples were stored at room temperature for 8 days and then analyzed. Analysis was done in all cases by extracting with two 50 ml portions of hexane and washing with two 25 ml portions of 70% ethanol and two 25 ml portions of acetonitrile saturated with hexane. The hexane extracts were evaporated to dryness and redissolved in 0.5 ml hexane. Five µl were injected for gas chromatography. Results of this experiment are given in Table 11.

TABLE 11

Preservation Study Results

Sample		µg/liter Coprostanol
#1	Preserved with H_2SO_4 - No storage	79.4
#2	Preserved with H_2SO_4 - No storage	90.6
#3	Stored at 4°C	73.0
#4	Stored at 4°C	84.0
#5	Preserved with H_2SO_4 - Stored at room temperature	82.0
#6	Preserved with H_2SO_4 - Stored at room temperature	111
#7	Not preserved - Stored at room temperature	30.9
#8	Not preserved - Stored at room temperature	26.0

Assuming the average value of samples #1 and #2 represents the true value of coprostanol concentration in the original sample, we can calculate recoveries for the various experiments.

Preserved by refrigeration

$$\% \text{ Recovery} = \frac{78.5}{85.0} \times 100 = 92.6\%$$

Preserved by addition of H_2SO_4

$$\% \text{ Recovery} = \frac{96.5}{85.0} \times 100 = 113\%$$

No preservation

$$\% \text{ Recovery} = \frac{28.4}{85.0} \times 100 = 33.4\%$$

CHAPTER VIII

FIELD SURVEYS

The field studies reported here have been undertaken to determine the applicability of free coprostanol as a useful indicator of water quality, and to determine any possible relationship between free coprostanol and biological indicators in water samples.

Experimental

Procedure

Surveys were made of two areas in the city of Gainesville, Florida. The first area surveyed was Sweetwater Creek—Payne's Prairie, and the second was the University of Florida Sewage Treatment Plant—Lake Alice. Samples were taken at locations shown on the maps (Figures 29 and 30). Numbers on the maps correspond to station numbers in Tables 12 and 13.

Every sample for chemical analyses was preserved with 1 ml conc. H_2SO_4 per liter. Extraction with hexane was done as soon as possible after sampling. Extraction and analysis were done in the manner detailed in Chapter III. None of the extracts were saponified to free the esters. It was found necessary to clean up by thin-layer chromatography all samples with a coprostanol content less than 1 µg/l. Thin-layer chromatography was performed using Eastman Chromagram Sheets. Development of the plates was with benzene.

Standard coprostanol and cholesterol were identified by spraying with a saturated solution of antimony trichloride and chloroform, as well as with ultraviolet light. Unknown locations corresponding to those of standards were cut out using scissors and redissolved in a small amount of hexane. Samples for microbiological tests were also taken at the same time as the chemical samples, and were analyzed the same day for total coliform, fecal coliform, and standard plate count.

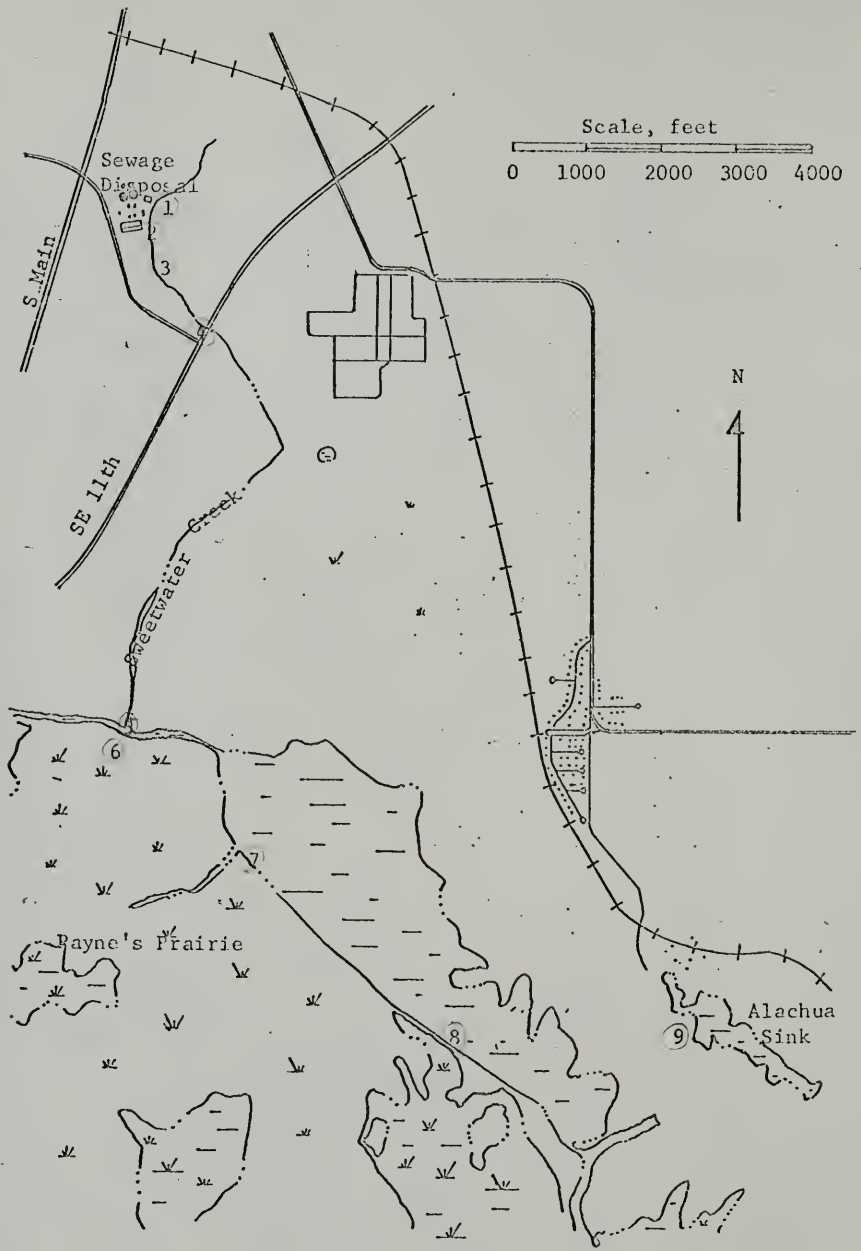


Fig. 29 - Sampling Stations, Sweetwater Creek-Payne's Prairie

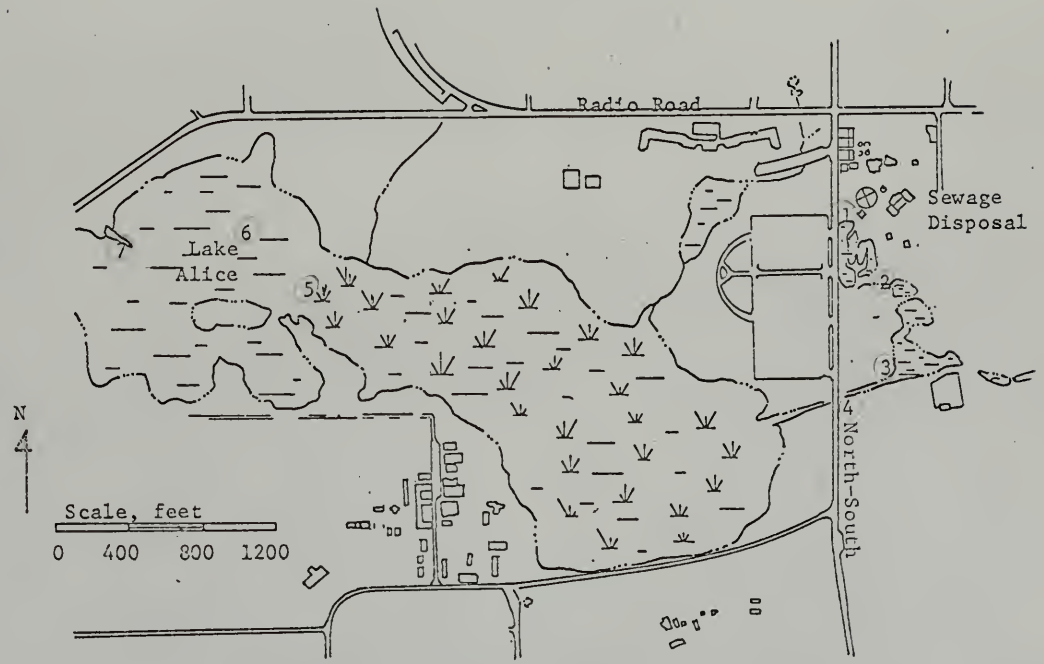


Fig. 30 - Sampling Stations, University of Florida Sewage Treatment Plant-Lake Alice

TABLE 12

Survey Data for Sweetwater Creek—Payne's Prairie
(March 9, 1971)

Station	Location	Volume of Sample, liters	Coprostanol µg/l	Total Coliform Number/100 ml	Fecal Coliform Number/100 ml	48 Hr. Standard Plate Count Number/100 ml
1	100 feet upstream from sewage outfall	1.0	0.027	6.4×10^4	80	3.8×10^6
2	Sewage effluent	0.5	65.0	26	0	7.4×10^3
3	300 feet downstream from sewage outfall	0.5	50.9	12	1	6.2×10^3
4	Highway 331 and Sweetwater Creek	1.0	39.2	72	0	2.8×10^4
5	Termination of Sweetwater Creek	1.0	24.3	1.12×10^3	0	1.12×10^6
6	120 feet inside Payne's Prairie	1.0	24.9	3.8×10^2	0	1.03×10^7
7.	Intersection of North-South and East-West Canals	1.0	0.033	1.47×10^4	61	6.9×10^6
8	250 yards south of Station 7	1.0	0.059	1.78×10^4	57	4.95×10^6
9	Alachua Sinkhole	1.0	0.061	3.38×10^4	15	3.35×10^6

TABLE 13

Survey Data for University of Florida Sewage Treatment Plant—Lake Alice
(March 23, 1971)

Station	Location	Volume of Sample, liters	Coprostanol µg/l	Total Coliform Number/100 ml	Fecal Coliform Number/100 ml	48 Hr. Standard Plate Count Number/100 ml
1	Effluent after chlorination	0.5	54	3.22×10^4	9	1.87×10^7
2	After oxidation pond	0.5	15	5.42×10^5	960	1.06×10^7
3	100 yards downstream from #2	1.0	9.9	5.55×10^5	1.37×10^3	4.0×10^7
4	Near underpass of canals in North-South Road	1.0	2.4	1.85×10^5	250	2.03×10^7
5	East end of Lake Alice	1.5	0.22	7.3×10^4	-	1.68×10^8
6	Middle of Lake Alice	1.5	0.014	1.37×10^4	96	1.46×10^8
7	West end of Lake Alice near out flow	1.5	0.022	1.17×10^2	66	1.34×10^7

CHAPTER IX

DISCUSSION OF RESULTS

Gas Chromatography

Since no extraneous peaks were found on any of the chromatograms of free coprostanol, and since the peaks obtained were sharp with only a small amount of tailing, there is no necessity to prepare the trimethylsilyl ether (TMS) derivatives of coprostanol prior to gas chromatography. This conclusion has also been reached by Bunch, who formerly prepared the TMS derivatives.[5,72]

Two conditions must be met in order to chromatograph free sterols. First, an all-glass column should be used, since metal columns have been known to cause breakdown of steroids. Copper, in particular, will catalyze the decomposition.[95] Stainless steel columns may give good results, but there must be a glass insert in the injection port to avoid steroid contact with very hot metal during flash evaporation. Second, all adsorptive sites on the solid support should be deactivated to avoid steroid decomposition. Deactivation is achieved by silanizing the solid support. Gas Chrom Q is an acid-washed, silanized support of diatomaceous earth.

In order to use peak height as a quantitative measure of coprostanol, there should be sufficient resolution of coprostanol from cholesterol, since cholesterol is invariably found with coprostanol

in the environment. Complete resolution was not necessary. Figure 9 shows that the required resolution of cholesterol was achieved by use of a 6 foot column. If one wishes to increase resolution of cholesterol and coprostanol, it can be done by increasing the length of the column, lowering the column temperature, lowering the percent liquid phase on the column, adjusting the flow rate of the carrier gas, or changing to a more selective liquid phase.[96]

The formation of the heptafluorobutyrate derivative of coprostanol evidently does not take place as easily as reported in the literature for plasma estrogen and plasma testosterone.[92,93,94] Klyne states that for reactions dependent on accessibility of x (esterification and hydrolysis of esters) equatorial reacts more rapidly than axial (see Figure 31).[97] Since the configuration of the OH group in coprostanol is axial, the reaction may need more drastic conditions than those reported in the literature for the estrogens and testosterone.

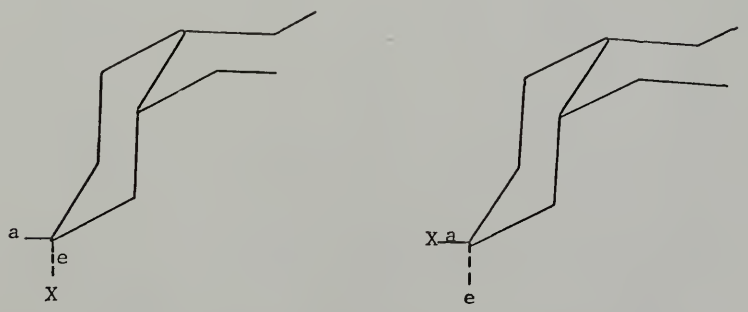


Fig. 31 - Equatorial and Axial Configurations of the Number 5 Position in 3β-steroids.

Extraction and Cleanup

N,N-dimethylformamide cannot be used to clean up the hexane extract, since some of the coprostanol was extracted into the dimethylformamide. Acetonitrile showed more promise of cleaning up the coprostanol extract. Indications were that very little of the coprostanol was removed by the acetonitrile. Since acetonitrile has a potential for extracting some of the interferents in the GLC analysis for coprostanol, a washing with acetonitrile was used in all subsequent analyses.

Saponification

The initial survey of the effect of saponification on the analysis of wastewaters showed no statistical difference between the saponified and unsaponified samples (experiment #1, Chapter VI). Since only one sample was taken from each source, three more experiments were performed, refining the experimental design. In these three experiments, 10 repetitions from each source were analyzed before and after saponification. It was found that saponification had a slight effect on the quantitative determination of coprostanol. However, the difference was slight, on the order of 10-15 percent. Such a slight difference does not seem to justify a three-hour saponification step in a procedure that takes four hours to complete the total analysis. Without saponification, analysis time was only one hour. Since the purpose of the analysis is to detect fecal pollution, it would seem that free coprostanol could do this as well as total coprostanol, including the esters of coprostanol. It appears

possible also, that the ratio of free coprostanol to total coprostanol is a constant, having a value of about 0.9, although this point needs further investigation.

The esterification of sterols in feces requires an enzyme system, probably of bacterial origin.[49] It is highly unlikely that this esterification of coprostanol could occur in the natural environment. The almost constant ratio of coprostanyl esters to free coprostanol found in sewage influent, trickling filter effluent, and activated sludge effluent indicates that not only is the ratio of coprostanyl esters to free coprostanol essentially constant but that they are biodegraded at the same rate. That is, the biodegrading organisms do not distinguish between coprostanyl esters and free coprostanol.

Microbial Degradation of Coprostanol

It is obvious from the results in Chapter VII that coprostanol decreases at a more rapid rate in sewage effluent taken before chlorination than in samples treated by chlorination. One explanation is that the disappearance of coprostanol is due to a biological degradation.

The apparent decrease in rate of disappearance of coprostanol with time in experiments 1 and 2 can be explained as due to the decreasing concentration of coprostanol with time. If the concentration of coprostanol is constantly becoming smaller with time, this means that the coprostanol as food supply for microorganisms is becoming more scarce. And the more scarce the coprostanol, the less likely a microorganism will encounter and biodegrade the coprostanol.

The curve of coprostanol versus time obtained in sample #1 does not fit a first order equation. Two factors may determine the rate change of coprostanol, coprostanol concentration and concentration of organisms capable of biodegrading coprostanol, since both are necessary for the biodegradation to occur.

Results obtained for indicator microorganisms can be divided into two categories, those obtained from chlorinated samples and those obtained from non-chlorinated samples. In general, the results obtained for chlorinated samples were more erratic, showing decreases after chlorination, followed by regrowth of organisms. Non-chlorinated effluents, on the other hand, showed a general decrease in numbers of organisms with time.

Both indicator microorganisms and coprostanol showed the same general trend for non-chlorinated samples, decreasing with time.

Chlorination affected both indicator microorganisms and coprostanol concentration, but in different ways. While indicator microorganisms showed an erratic pattern of decrease followed by regrowth, coprostanol concentration decreased slowly with time. It would appear from these experiments that correlation between indicator microorganisms and coprostanol may be found where the sewage effluent is not chlorinated. Where chlorination is practiced, since the effect of chlorine on microorganisms and coprostanol is different, no correlation may be expected.

Field Surveys

The concentration of coprostanol in Sweetwater Creek and Payne's Prairie was found to decrease with increase in distance from the source of pollution, the City of Gainesville Sewage Treatment Plant.

High concentrations of coprostanol were found in all samples taken in Sweetwater Creek downstream from the treatment plant. Very low concentrations of coprostanol were found in samples taken upstream from the treatment plant as well as for samples taken well inside Payne's Prairie.

No apparent relationship between coprostanol and any of the microbiological indicators could be found. Chlorination had the effect of lowering appreciably the concentration of microorganisms, without apparently affecting the coprostanol concentration. The result was that low counts of microorganisms were found near the plant, where the water was hardly of good quality, while high counts of microorganisms were found at distant points where one would have expected water quality to have been improved. High counts of coliform organisms found in Payne's Prairie may have been due to the very high aquatic bird population (estimated 15,000-20,000 ducks, 1,000 Sand Hill Cranes and 3,000 egrets and herons)[97] found there during the winter months. One duck is said to contribute 11,000 Fecal Coliform per 24 hours while a man contributes 2,000 Fecal Coliform.[98] Thus, a 15,000-20,000 duck population alone would correspond to the Fecal Coliform production of a human population of 82,500 to 110,000.

As in the first survey, the concentration of coprostanol in the University of Florida Sewage Treatment Plant—Lake Alice survey was found to decrease with increasing distance from the source of pollution. There was a significant decrease in coprostanol as the sewage effluent passed through the oxidation pond (54 µg/l to 15 µg/l). Fairly high concentrations of coprostanol were found in all samples taken from the stream leading into Lake Alice. However, very low concentrations of coprostanol were found in Lake Alice itself. Only the east end of Lake Alice could be said to contain significant amounts of coprostanol. No difficulty was encountered in analyzing for free coprostanol in the environment.

Once again chlorination had the effect of decreasing significantly the number of microorganisms to be found in the water in samples taken near the sewage outfall. There was an apparent regrowth followed by a decrease in numbers of coliform organisms. This effect of regrowth is apparently a common phenomenon. As a result of the changing character of the coliform organisms, no relationship could be found between these microbiological indicators and coprostanol levels.

It would appear from the above study that no simple relationship between microbiological indicator organisms and coprostanol exists, especially if the sewage effluent is highly chlorinated. Coprostanol would appear to be a better indicator of fecal pollution than coliform in those waters which receive highly chlorinated effluent. Coprostanol concentrations in such cases would be high, where coliform organisms may be quite low and a danger due to either resistant pathogenic bacteria or viruses may be present.

High aquatic bird populations may cause high coliform counts in water,[98] while no real danger exists. Although some diseases may be transmitted from birds to man,[99] none of these diseases are normally transmitted by the water route. None of the major water borne diseases listed in *Control of Communicable Diseases in Man*[100] are given as being caused by birds as reservoirs of the disease.

Preservation of Samples

Preservation of stored samples is absolutely necessary. The studies reported in Chapter VII show that only 33.4 percent of coprostanol was recovered after 8 days' storage without preservation. Data in Chapter VII also indicate that preservation may be accomplished either by the addition of 1 ml of H_2SO_4 per liter of water or by storing the samples at 4°C.

CHAPTER X

SUMMARY AND CONCLUSIONS

This study has shown that the analysis of coprostanol can be greatly simplified. For most samples (those with concentrations of coprostanol greater than 1 µg/liter) it is not necessary to clean up with thin-layer chromatography. A simple extraction with hexane, followed by washings with 70 percent ethanol and acetonitrile saturated with hexane is all that is necessary before analysis by gas-liquid chromatography.

It seems unnecessary to saponify samples prior to GLC. Saponification is a very time-consuming technique and free coprostanol, which accounts for approximately 90 percent of total coprostanol, can be used just as meaningfully for the purpose of detecting fecal contamination of water.

Coprostanol may be chromatographed as the free sterol rather than the TMS derivative used previously. It is necessary to use an acid-washed, silanized support, such as Gas Chrom Q, along with an all-glass column when chromatographing the free sterol.

The disappearance of coprostanol in the aqueous environment appears to be due to the biodegradation of coprostanol by microorganisms. Even the removal of coprostanol in the activated sludge process appears to be principally due to biodegradation, although physical removal is also of importance. This process of biodegradation is slowed down by

chlorination of wastewater. In non-chlorinated wastewater, the process of biodegradation occurs rapidly at first, but the rate of biodegradation decreases with time. Although the concentration of coprostanol decreases continuously with time, indicator microorganisms were found to fluctuate with passage of time, showing periods of decrease and increase. Aftergrowth, or regrowth, has been shown to occur with the non-fecal portion of the total coliform group.[101] In one study on the American River below Sacramento, California, no regrowth of fecal coliforms was found to occur below the outfall of a highly treated wastewater.[102] Apparently, no studies on regrowths of pathogenic organisms have been reported.

Environmental surveys show that free coprostanol can be detected easily at locations near the source of pollution, but that detection becomes more difficult at distant locations having sample concentrations below 1-2 μg/liter. These low concentrations require TLC cleanup. No correlation between microbiological indicators and coprostanol was found. While coprostanol was found to decrease in concentration with increasing distance from the source of pollution, counts for microbiological indicators were found to fluctuate with the high counts in Payne's Prairie possibly due to the large aquatic bird population.

An ideal indicator of fecal pollution should have several characteristics.[103] These, along with the ability of coprostanol to fulfill them, are:

1. It should yield uniform results in all types of water.

Coprostanol analysis should give identical results in all types of water, although some waters may require additional cleanup by TLC.

2. The indicator should never be present in bacteriologically safe water.

   Coprostanol, since its only source is the feces of mammals, should not be present in bacteriologically safe water. It would only be present in bacteriologically safe water if none of the individuals contributing coprostanol contributed pathogenic organisms.

3. The concentration of indicator should increase in proportion to the amount of sewage pollution.

   Coprostanol fulfills this requirement since the concentration of coprostanol is highest in fresh untreated sewage. Treated sewage, or diluted sewage would have a lower concentration of coprostanol, the concentration decreasing with increasing treatment or dilution.

4. It should have a high degree of specificity. No other substance should give positive results.

   It is unlikely that another peak would occur with the same retention time as coprostanol. If there were doubt in the identification of a peak as coprostanol, it would be possible to run coprostanol on a second column for positive identification.

5. It should be harmless to man.

   Coprostanol is harmless to man.

6. The indicator should survive in water longer than pathogens.

   No studies comparing survival times for pathogens and coprostanol have been reported.

7. It should disappear from the water supply rapidly after pathogens are removed.

   No studies to determine this have been reported.

8. It should be a constant occurrence in human feces.

   Coprostanol is consistently present in human feces.

9. It should be consistently present in sewage and polluted waters.

   Coprostanol is consistently present in sewage and waters polluted with sewage.

10. It should be detectable by a simple test.

    Coprostanol is easily detectable using a simple extraction procedure and GLC. For low concentrations a cleanup by TLC may be necessary.

11. It should be detectable by a rapid test.

    A single sample may be analyzed for coprostanol in one hour if no cleanup by TLC is necessary. Two hours are required for samples needing TLC cleanups.

The test for coprostanol seems particularly valuable in at least three instances where microbiological indicators give poor results. Sewage that is heavily chlorinated shows low readings for coliform organisms, while the dangers from pathogens, including viruses, may still be present. In such cases, coprostanol, which does not appear to be affected by concentrations of chlorine used in disinfection, may give better information about the potential danger involved.

The National Technical Advisory Subcommittee on Public Water Supplies has stated that "In the past the coliform test has been the principal criterion of suitability of raw-water sources for public water supply. The increase in chlorination of sewage treatment plant effluents distorts this criterion by reducing coliform concentrations without removing many other substances which the defined water treatment plant is not well equipped to remove. It is essential that raw-water sources be judged as to suitability by other measures and criteria than coliform organism concentrations."[104] Coprostanol would seem to be one criterion that would tell more about the suitability of raw water for public water supply.

Another case where coprostanol analysis may give better results than coliform analysis is those samples which are not able to be analyzed for several days after sampling. Coliform results have been shown to vary significantly with sample transit time, and it has been concluded that the validity of data obtained in processing samples over 48 hours old is highly questionable.[105] Coprostanol analyses may be done after passage of a number of days, since the addition of H_2SO_4 will kill the bacteria responsible for biodegrading coprostanol, thus preserving the coprostanol during transit to the laboratory.

A third case where coprostanol may be a more convenient indicator than coliform is where a rapid answer is needed concerning the quality of a particular water sample. Coliform analysis takes at least 24 hours to obtain results, while analysis for a single sample of coprostanol may be done in one hour. In certain emergency situations, then, coprostanol analysis may be more useful than coliform analysis.

The fecal coliform test is said to be applicable to investigations of stream pollution, raw-water sources, sewage treatment systems, bathing waters, sea waters and general water quality monitoring.[28] The coprostanol test could also be applied to the same types of investigations.

Further studies are needed to determine what levels of coprostanol should serve as water quality standards for various uses. Until such studies are made, a possible guide would be the comparison given by Bunch[5] that human feces average 2×10^9 fecal coliform bacteria and 2×10^9 nanograms of coprostanol per day per capita. This value for coprostanol can be seen to be reasonable since an average value

for sewage volume would be 100 gpd/capita and raw sewage generally has a concentration of about 600 µg/liter. This would correspond to 2.2 grams or 2.2×10^9 nanograms of coprostanol per day per capita.

Recommendations for recreation waters for primary contact recreation are a mean fecal coliform concentration not greater than 200/100 ml during a 30-day period, nor should more than 10 percent of total samples during any 30-day period exceed 400/100 ml.[106] These values correspond to 2 and 4 µg/liter for coprostanol. Secondary contact recreation waters are recommended to have an average fecal coliform concentration not exceeding 2000/100 ml and a maximum of 4000/100 ml, except in specified mixing zones adjacent to outfalls.[106] These values correspond to 20 and 40 µg/liter of coprostanol.

Permissible surface water fecal coliform criteria for public water supplies are given as 2000/100 ml, while desirable criteria are less than 20/100 ml.[106] These values correspond to coprostanol concentrations of 20 µg/liter and 0.2 µg/liter respectively.

No recommendations are given for fecal coliform concentrations in shellfish waters, but recommendations are given for total coliform. These recommendations are quite strict, giving values of 70/100 ml as the mean acceptable concentration and 230/100 ml as being unacceptable if found in more than 10 percent of the samples.[107] If we consider fecal coliform counts to be one-fifth of total coliform counts, the standard for coprostanol would be 0.14 µg/liter mean acceptable and 0.46 µg/liter unacceptable if occurring in more than 10 percent of the samples.

Irrigation waters should have a mean value for fecal coliform not in excess of 1000/100 ml and no sample should have a concentration

in excess of 4000/100 ml. These values correspond to coprostanol concentrations of 10 and 40 µg/liter respectively.

The stream surveys by Tabak *et al.* of the Missouri, Mississippi, and Ohio River Basins[72] show the value of coprostanol in characterizing water quality. Surveys such as these, performed over the years, would enable absolute evaluation of the effectiveness of municipal sewage treatment in a given basin.

While the coprostanol test does not supplant the fecal coliform test, it does provide the investigator with another tool to better characterize water quality.

APPENDIX

APPENDIX I

Although it has been stated[5,72] that coprostanol is produced from cholesterol exclusively in the mamalian intestine, one author has claimed detecting coprostanol in the feces of chickens.[108] For this reason, a supplemental experiment analyzing feces of chickens was performed.

Experiment

Eighteen grams of fresh chicken feces were taken and extracted with 50 ml of acetone. The acetone was separated from the solid feces by filtering, and then diluted with 50 ml water. The mixture was added to a separatory funnel and 30 ml hexane was added.

The hexane layer, containing the sterols, was separated from the acetone-water layer. The hexane was washed twice with 15 ml of 70 percent ethanol and the volume of hexane was reduced on a flash evaporator to approximately 13 ml. Aliquots of the hexane were chromatographed and the results are shown in Figure 32. The chromatograph shows that while cholesterol was found, there was no indication of coprostanol.

Fig. 32 - Chromatogram of Extract from Chicken Feces

REFERENCES

1. McGauhey, P. H., "Engineering Management of Water Quality." McGraw Hill (1968).

2. Senate Select Committee on National Water Resources (Jan. 30, 1961).

3. McCallum, G. E., "Government Functions in Pollution Control. Joint Discussion. Federal Activities." *JAWWA*, 53, 851 (1961).

4. Standard Methods for the Examination of Water and Wastewater. 13th edition, 684 (1971).

5. Murtaugh, J. J. and Bunch, R. L., "Sterols as a Measure of Fecal Pollution." *JWPCF*, 39, 404-409 (1967).

6. Smith, L. L. and Gouron, R. E., "Sterol Metabolism-VI. Detection of 5β-cholestan-3β-ol in Polluted Waters." *Water Research*, 3, 141-148 (1969).

7. Black, A. P., Singley, J. E., and Nordstrand, E., Unpublished Work. Univ. of Fla., Gainesville.

8. Sanitary Significance of Fecal Coliforms in the Environment. U.S. Dept. of the Interior, Federal Water Pollution Control Admin. Pub. WP-20-3.

9. U.S. Public Health Service Drinking Water Standards. (1962).

10. Geldreich, E. E., "Applying Bacteriological Parameters to Recreational Water Quality." *JAWWA*, 62, 113-120 (1970).

11. Sharman, J. M., "The Streptococci." *Bact. Rev.*, 1, 3-97 (1937).

12. Hajna, A. A. and Perry, C. A., "Comparative Study of Presumptive and Confirmative Media for Bacteria of the Coliform Group and for Fecal Streptococci." *Am. J. Pub. Health*, 33, 550-556 (1943).

13. Winter, C. E. and Sandholzer, L. A., "Studies on the Fecal Streptococci." Fishery Leaflet No. 201. Washington, D. C. Fish and Wildlife Service. Dept. of Interior.

14. Escherich, T., "Die Darmbakterien des Neugeborenen und Sauglings." *Fortschr. der Med.*, 3, 515 (1885).

15. Rogers, L. A., *et al.*, "The Characteristics of Bacteria of the Colon Type Found in Bovine Feces." *J. Infectious Diseases*, 15, 99 (1915).

16. Parr, L. W., "The Occurrence and Succession of Coliform Organisms in Human Feces." *Am. J. Hyg.*, 27, 67 (1938).

17. Foote, H. B., "The Possible Effects of Wild Animals on the Bacterial Pollution of Water." *JAWWA*, 29, 72 (1937).

18. Rogers, L. A., *et al.*, "The Characteristics of Bacteria of the Colon Type Occurring on Grains." *J. Infectious Diseases*, 17, 137 (1915).

19. Taylor, C. B., "The Ecology and Significance of the Different Types of Coliform Bacteria Found in Water." *J. Hyg.*, 42, 23 (1942).

20. Parr, L. W., "Viability of Coli-aerogenes Organisms in Culture and in Various Environments." *J. Infectious Diseases*, 60, 291 (1937).

21. Platt, A. E., "The Viability of Bacteria Coli and Bacteria Aerogenes in Water: A Method for the Rapid Enumeration of these Organisms." *J. Hyg.*, 35, 437 (1935).

22. Chen, C. C. and Retger, L. F., "A Correlation Study of the Colon-Aerogenes Group of Bacteria, with Special Reference to the Organisms Occurring in the Soil." *J. Bacteriol.*, 5, 253 (1920).

23. Bardsley, D. A., "The Distribution and Sanitary Significance of E. Coli, B. lactis aerogenes, and Intermediate Types of Coliform Bacilli in Water, Soil, Feces, and Ice Cream." *J. Hyg.*, 34, 38 (1934).

24. Frank, N. and Skinner, C. E., "Coli-aerogenes Bacteria in Soil." *J. Bacteriol.*, 42, 143 (1941).

25. Anyot, J. A., "Is the Colon Bacillus a Normal Habitant of Intestines of Fishes?" *Amer. J. Pub. Health*, 27, 400 (1901).

26. Evelyn, T. P. T. and McDermott, L. A., "Bacteriological Studies of Fresh-Water Fish. I. Isolation of Aerobic Bacteria from Several Species of Ontario Fish." *Can. J. Microbiol.*, 7, 375 (1961).

27. Gibbons, N. E., "Lactose Fermenting Bacteria from the Intestinal Contents of Some Marine Fishes." *Contribs. Can. Biol. and Fisheries (N.S.)*, 8, 291 (1934b).

28. *Standard Methods for the Examination of Water and Wastewater.* APHA, AWWA, and WPCF, New York. 13th edition, 662 (1971).

29. Laufer, S., "Brewing Water." 3rd Maritime Conf. Master Brewers Assoc. of America. (Oct. 12-14, 1944).

30. Rudolf, Z., "Principles of the Determination of the Physical and Chemical Standards of Water for Drinking, Industrial, and Domestic Purposes." *Gay i Woda.*, 10, 57 (1930); Water Pollution Abs. 4 (Mar. 1931).

31. Anon., "European Standards for Drinking-Water." World Health Organization, Geneva (1961).

32. Sawyer, C. N., *Chemistry for Sanitary Engineers.* McGraw-Hill, 257 (1960).

33. Prescott, S. C., Winslow, C. E. A., and McCrady, M. H., *Water Bacteriology.* John Wiley and Sons, 6th edition, 239. Quote attributed to Dr. T. M. Drown. (1947).

34. Bunch, R. L. and Ettinger, M. B., "Water Quality Depreciation by Municipal Use." *JWPCF,* 36, 1411-14 (1964).

35. Kukchek, G. J. and Edwards, G. P., "Uric Acid as a Measure of Water Pollution." *JWPCF,* 34, 376-89 (Apr. 1962).

36. O'Shea, J. and Bunch, R. L., "Uric Acid as a Pollution Indicator." *JWPCF,* 37, 1444-46 (1965).

37. Baldwin, E., Introduction to Comparative Biochemistry. 3rd edition, Cambridge Univ. Press. (1949).

38. Gould, R. G. and Cook, R. P., "The Metabolism of Cholesterol and Other Sterols in the Animal Organism." In Cholesterol. (Edited by R. P. Cook) Academic Press, 289-92, New York.

39. Seylers, H., *Z. Physiol. Chem.*, 348, 1688 (1967).

40. Danielsson, H., "Present Status of Research on Catabolism and Excretion of Cholesterol." *Adv. in Lipid Res.*, 1,335 (1963).

41. Cook, R. P., *et al.*, "Cholesterol Metabolism 7, Cholesterol Adsorption and Excretion in Man." *Biochem. J.*, 62, 225 (1956).

42. Aylward, F. and Wills, P. A., "Lipid Excretion 1. Sterols and Sterol Esters." *Brit. J. Nutr.*, 16, 339 (1962).

43. Rosenfeld, R. S., "The Isolation of Coprostanol from Sterol Esters of Human Feces." *Arch. Biochem. Biophys.*, 108, 384 (1964).

44. Snog-Kjaer, A., Prange, I. and Dam, H., "Conversion of Cholesterol into Coprostanol by Bacterial in Vitro." *J. Gen. Microbiol.*, 14, 256 (1956).

45. Coleman, D. L. and Bauman, C. A., "Intestinal Sterols. V. Reduction of Sterols by Intestinal Microorganisms." *Arch. Biochem. Biophys.*, 72, 219 (1957).

46. Rosenfeld, R. S., *et al.*, "The Transformation of Cholesterol to Coprostanol." *J. Biol. Chem.*, 211, 301 (1954).

47. Rosenfeld, R. S., *et al.*, "The Transformation of Cholesterol-3d to Coprostanol-d. Location of Deuterium in Coprostanol." *J. Biol. Chem.*, 222, 321 (1956).

48. Rosenfeld, R. S. and Gallagner, T. F., "Further Studies of the Biotransformation of Cholesterol to Coprostanol." *Steroids*, 4, 515 (1964).

49. Rosenfeld, R. S., "Biosynthesis of Coprostanyl Esters." *Arch. Biochem. Biophys.*, 112, 621 (1965).

50. Eneroth, P., *et al.*, "Identification and Quantification of Neutral Fecal Steroids by Gas-Liquid Chromatography and Mass Spectrometry: Studies of Human Excretion During Two Dietary Regimens." *J. Lipid Res.*, 5, 245 (1964).

51. Grundy, S. M., *et al.*, "Quantitative Isolation and Gas-Liquid Chromatographic Analysis of Total Fecal Bile Acids." *J. Lipid Res.*, 6, 397 (1965).

52. Miettinen, T. A., *et al.*, "Quantitative Isolation and Gas Liquid Chromatographic Analysis of Total Dietary and Fecal Neutral Steroids." *J. Lipid Res.*, 6, 411 (1965).

53. Spritz, N., *et al.*, "Sterol Balance in Man as Plasma Cholesterol Concentrations are Altered by Exchanges of Dietary Fats." *J. Clin. Inves.*, 44, 1482 (1965).

54. "Hormones and Atherosclerosis." Academic Press, 157 (1959).

55. Rosenfeld, R. S. and Hellman, L., "The Relation of Plasma and Bilary Cholesterol for Bile Acid Synthesis in Man." *J. Clin. Invest.*, 38, 1334 (1959).

56. Fieser, L. F. and Fieser, M., "Steroids." Reinhold Publishing Company (1959).

57. Handbook of Chemistry and Physics. 40th edition. C-259.

58. Saad, H. Y. and Higuchi, W. I., "Water Solubility of Cholesterol." *J. of Pharmaceutical Sciences*, 54, 1205-1206 (1965).

59. Brown, H. H., *et al.*, "Rapid Procedure for Determination of Free Serum Cholesterol." *Anal. Chem.*, 26, 397 (1954).

60. Zak, B., *et al.*, "Determination of Serum Cholesterol." *Amer. J. Med. Tech.*, 23, 283 (1957).

61. Gerson, T., "The Determination of Cholesterol and Coprosterol in Fecal Lipids." *Biochem. J.*, 77, 446 (1960).

62. Jorgensen, K. H. and Dam, H., "An Ultra Micromethod for the Determination of Total Cholesterol in Bile Based on the Tschugaeff Color Reaction." *Acta Chemica Scandinavica*, 11, 1201-1208 (1957).

63. Smith, L. L., *et al.*, "Sterol Metabolism. IV. Microbial Disposition of 5β-Cholestan-3β-ol." *Lipids*, 3, 301-306 (1968).

64. Cook, R. P., *et al.*, "Cholesterol Metabolism 7. Cholesterol Absorption and Excretion in Man." *Biochem. J.*, 62, 225 (1956).

65. Aylward, F. and Wills, P. A., "Lipid Excretion 1. Sterols and Sterol Esters." *Brit. J. Nutr.*, 16, 339 (1962).

66. Danielsson, H., "Present Status of Research on Catabolism and Excretion of Cholesterol." *Advan. Lipid Res.*, 1, 335 (1963).

67. Rosenfeld, R. S., "The Isolation of Coprostanol from Sterol Esters of Human Feces." *Arch. Biochem. Biophys.*, 108, 384 (1964).

68. Rosenfeld, R. S., "Biosynthesis of Coprostanyl Esters." *Arch. Biochem. Biophys.*, 112, 621 (1965).

69. Vandenheuvel, W. J. A., Sweeley, C. C. and Horning, E. C., "Separation of Steroids by Gas Chromatography." *J. Am. Chem. Soc.*, 82, 3481 (1960).

70. Wells, W. W. and Makita, M., "The Quantitative Analysis of Fecal Neutral Sterols by Gas-Liquid Chromatography." *Anal. Biochem.*, 4, 204 (1962).

71. Luukkainen, T., *et al.*, "Gas Chromatographic Behavior of Trimethylsilyl Ethers of Steroids." *Biochem. Biophys. Acta.*, 52, 599 (1961).

72. Tabak, H. H., Bloomhuff, R. N. and Bunch, R. L., "Steroid Hormones as Water Pollutants. II. The Use of Coprostanol as a Positive Marker of Domestic and Run Off Pollution." To be published in *Development in Industrial Microbiology*, 13 (1972).

73. Sohngen, N., *Zentralbl, f. Bakt and Parasitenk,* II, 37, 595 (1913).

74. Turfitt, G. E., "Microbiological Agencies in the Degradation of Steroids. II Steroid Utilization by the Microflora of Soils." *J. Bact.,* 54, 557 (1947).

75. Haag, F., *Archev. f. Hygiene,* 97, 28 (1926).

76. Haag, F., *Zent. Bakteriol. Parasitenk II.,* 71, 1 (1927).

77. Tak, J. D., *Antonie van Leeuwenhoek. J. Microbiol. Serol.,* 8, 32 (1942).

78. Schatz, A., *et al., J. Bact.,* 58, 117 (1949).

79. Turfitt, G. E., "The Microbiological Degradation of Steroids I. The Cholesterol Decomposing Organisms of Soils." *Biochem. J.,* 37, 115-117 (1943).

80. Turfitt, G. E., "The Microbiological Degradation of Steroids. II Oxidation of Cholesterol by Proactinomyces spp." *Biochem. J.,* 38, 494-496 (1944).

81. Turfitt, G. E., "Microbiological Agencies in the Degradation of Steroids I. The Cholesterol Decomposing Organisms of Soils." *J. Bact.,* 47, 487 (1944).

82. Sobel, H. and Plant, A., "The Assimilation of Cholesterol by Mycobacterium Smegmatis." *J. Bacteriol.,* 57, 377 (1949).

83. Wainfan, *et al.,* "Metabolism of Cholesterol by Intestinal Bacteria in Vitro." *J. Bio. Chem.,* 207, 843 (1954).

84. Arima, K., *et al.,* "Microbial Transformations of Sterols Part I. Decomposition of Cholesterol by Microorganisms." *Agr. Biol. Chem.,* 33, 1636-39 (1969).

85. Smith, L. L. and Mathews, W. S., 7th Int'l Congr. Biochem., Tokyo, Japan.

86. Jeffrey, L. M., *JAOCS,* 43, 211-14 (1966).

87. Pomeroy, R. and Wakeman, C. M., "Determination of Grease in Sewage, Sludge and Industrial Wastes." *Indust. Engng. Chem. Analyt. Ed.,* 13, 795-801 (1941).

88. Knechtges, O. J., *et al.,* "The Lipids of Sewage Sludges." *Sewage Wks. Jr.,* 6, 1082-1093 (1934).

89. Loehr, R. C. and Higgins, G. C., "Comparison of Lipid Extraction Methods." *Int. J. Air Wat. Poll.*, 9, 55-57 (1965).

90. Mills, P. A., *et al.*, "Rapid Methods for Chlorinated Pesticide Residues in Non-fatty Foods." *JAOAC*, 46, 186 (1963).

91. Burchfield, H. P., "Drug and Pesticide Analysis by Gas Chromatography in Toxicology, Metabolism and Residue Analysis Studies." Presented at Course on Biomedical and Clinical Applications of Gas Chromatography, Washington University, Saint Louis, Missouri (1969).

92. Clark, S. J. and Wotiz, H. E., "Separation and Detection of Nanogram Amounts of Steroids." *Steroids*, 2, 535 (1963).

93. Wotiz, H. H., *et al.*, "Gas Chromatographic Measurement of Plasma Estrogens Using an Electron Capture Detector." *Steroids*, 10, 127 (1967).

94. Exley, D., "Ultramicro Determination of Plasma Testosterone by Electron-Capture Detection of Testosterone Diheptafluorobutyrate." *Biochem. J.*, 107, 285 (1968).

95. Arnold, J. E. and Fales, H. M., "A Comparison of Column and Connector Materials in the Gas Chromatography of Sensitive High Molecular Weight Compounds." *J. Gas Chrom.*, 3, 131 (1965).

96. Levy, R., "High Resolution Gas Chromatography." Presented at course on Biomedical and Clinical Applications of Gas Chromatography, Washington University, Saint Louis, Missouri (1969).

97. Personal Communication. Mr. Jerry Brown, Game and Freshwater Fish Commission. State of Florida, Gainesville, Florida.

98. Geldrich, E. E., "Type Distribution of Coliform Bacteria in the Feces of Warm Blooded Animals." *JWPCF*, 34, 295 (1962).

99. Hull, T. G., "Diseases Transmitted from Animals to Man." Charles C. Thomas Publisher, 4th edition, 640-643 (1955).

100. Control of Communicable Diseases in Man. 11th edition, Abram S. Benenson Editor. Amer. Pub. Health Assoc. (1970).

101. Geldreich, E. E., Fecal Coliform Concepts in Stream Pollution. Water and Sewage Works Reference Number: R-9: to R-110 (1967).

102. Deaner, D. G. and Kerri, K. D., "Regrowth of Fecal Coliforms." *JAWWA*, 61, 465-468 (1969).

103. Fox, J. L., Department of Environmental Engineering, University of Florida, Gainesville, Florida. Personal Communication.

104. National Technical Advisory Committee Report. Raw-Water Quality Criteria for Public Supplies. *JAWWA*, 61, 33-138 (1969).

105. Geldreich, E. E., "Application of Bacteriological Data in Potable Water Surveillance." *JAWWA*, 63, 225-229 (1971).

106. Report of the Committee on Water Quality Criteria. Fed. Wat. Pol. Cont. Admin. (1968).

107. Lehninger, A. L., "Biochemistry." Worth Publishers (1970).

108. Hoffman, A., "Studies on the Suitability of Coprostanol as an Indicator of Fecal Pollution in Water." Dissertation presented to the Graduate School of the University of Massachusetts (1970).

BIOGRAPHICAL SKETCH

Cliff Joseph Kirchmer was born on November 28, 1939, at St. Louis, Missouri. He was graduated from St. Louis University High School in June, 1957 and went on to receive the degree of Bachelor of Science with a major in chemistry from St. Louis University in 1962. He taught chemistry and physics at Palmetto Senior High School in Miami, Florida from 1963 until 1965. In April of 1966 he received the degree of Master in the Science of Teaching with a major in chemistry from the University of Florida. From 1966 until 1968 he served as a Peace Corps volunteer in Chile, teaching chemistry at the Catholic University and the Federico Santa María Technical University. He pursued his work toward the degree of Doctor of Philosophy from September, 1968 until the present.

Cliff Joseph Kirchmer is married to the former María Luisa Canessa Delgadillo, and is the father of a son, James Lawrence. He is a member of the American Chemical Society and the American Water Works Association.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

J. Edward Singley, Chairman
Professor of Environmental Engineering

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Patrick L. Brezonik
Associate Professor of Environmental Engr.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jackson L. Fox
Assistant Professor of Environmental Engr.

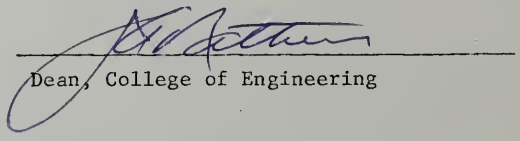
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Thomas deS. Furman
Professor of Environmental Engineering

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James D. Winefordner
Professor of Chemistry

This dissertation was submitted to the Dean of the College of Engineering and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.



Dean, College of Engineering

Dean, Graduate School